

THE PREDICTION OF HORMONE-DEPENDENCE IN MAMMARY CANCER

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## ABSTRACT

### The Prediction of Hormone-Dependence in Mammary Cancer.

A review has been made of the literature concerning clinical response rates to endocrine therapies for advanced mammary cancer and the need for a predictor of response has been identified. Various postulated predictors of response have been critically reviewed and Oestrogen Receptor (E/R) status has been identified as the best, currently available, approach. The main limitations of E/R status are, firstly, the poor predictive value of a positive result and, secondly, the complex nature of current techniques for its determination.

Two approaches to the prediction of hormone-dependence, aimed at overcoming these limitations of E/R status, have been investigated. Biochemical and histochemical systems for the identification of tissue peroxidase, a postulated, alternative marker for oestrogen-dependence, have been established. Peroxidase levels have been measured in a range of normal rat tissues, in rat mammary tumour models of hormone-dependent and -independent growth and in human mammary tumours and have been compared with levels of E/R and also of Progesterone Receptor (Pg/R). A relationship between peroxidase activity and hormone-dependence has been confirmed but the predictive value of peroxidase determination appears to be inferior to that of E/R assay.

The feasibility of a histochemical approach to the identification of E/R has been investigated. The effects of histochemical processing, including methods of tissue fixation, on E/R activity have been studied using tritiated oestradiol as tracer. Conjugates of oestradiol with tracers which can be visualised under the light, or fluorescence, microscope have been synthesised and evaluated. It has been demonstrated that histochemical processing results in considerable losses of detectable E/R activity and that oestradiol labelled with histochemical tracers has a very small ability to bind to E/R. Nevertheless, uptake of labelled oestradiol by oestrogen-target tissues, including some human mammary cancers, has been demonstrated and such uptake may be related to bona fide E/R activity.

DECLARATION OF ORIGINALITY.

I declare that the text of this thesis has been composed entirely by myself. Also, I have undertaken all of the experimental work described within, with the exception of some technical help in the preparation and purification of tritium-labelled steroid solutions and in the cutting of histological sections.

The experimental work described in this thesis has, in part been published in the following paper:

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PART I

INTRODUCTORY REVIEW

# I. THE CONCEPT OF HORMONE-DEPENDENCE IN MAMMARY CANCER

The origin of the concept that malignant cells are not always autonomous but may retain sensitivity to the same hormonal influences that govern the behaviour of the parent tissue is credited to Charles Huggins and Peyton Rous. In 1966 they were jointly awarded the Nobel Prize in medicine and physiology in recognition of the far-reaching implications of the concept they had evolved. Endocrine ablation had been successfully undertaken for the control of advanced malignancy since the late 19th century but its basis had been purely empirical, and it was the classic experimental work of Huggins which led to an understanding of the nature of the relationship between the gonads and certain cancers. In 1940, Huggins and Clark described studies into the effects of castration and of the administration of hormones on hyperplastic prostate in the experimental dog. Their observations led them to investigate the effects of orchidectomy on prostatic cancer in man. They found a rewarding rate of response (Huggins et al., 1941).

In a lucid and enthusiastic review of "Advances in Cancer Research", Rous (1947) revealed his insight into the far-reaching implications of Huggins' laboratory and clinical observations:

"The significance of this discovery far transcends its practical application; for it means that thought and endeavour in cancer research have been misdirected in consequence of the belief that tumour cells are anarchic.....No one had realised that neoplastic cells, making such attempts at differentiation, might also respond, in line of duty, to normal, physiological influences such as the hormones exert."

This concept of hormone dependence was soon applied to the behaviour of mammary, as well as prostatic cancer. Years of empiricism had suggested, and, more recently, laboratory studies in cell and tissue culture (Borthwick

and Smellie, 1975; Montessori et al., 1977; Lippman and Bolan, 1975; Welsch and Rivera, 1972; Scott et al., 1979) have confirmed, that a proportion of human and experimental cancers exhibit growth-dependence on oestrogens.

Dependence of mammary cancers on hormones other than oestrogen has been postulated and, of these hormones, prolactin has stimulated most interest. The results of cell and tissue culture work (Montessori et al., 1977) would suggest that a proportion of human breast cancers exhibit dependence on prolactin and other hormones in vitro. It is well-proven that experimental rodent cancers are prolactin dependent, but clinical experience in humans would indicate that prolactin exerts little influence on tumour behaviour in vivo. Indeed, McMillan et al. (1977) have shown that serum prolactin may rise after hypophysectomy (perhaps due to secretion by residual pituitary stalk), and yet tumour regression may still occur. The available data on prolactin and breast cancer have been comprehensively reviewed by Smithline et al. (1975).

From the clinician's viewpoint, it may be considered that oestrogens are the only hormones which have been clearly shown to influence the behaviour of human mammary cancer in vivo. Therefore, the forms of endocrine manipulation currently practised for the control of advanced disease are largely aimed either at reducing the amount of circulating oestrogen available to the tumour cell, or at "blocking" the interaction of oestrogen with the cell.

## II. ENDOCRINE THERAPIES AVAILABLE FOR THE CONTROL OF ADVANCED BREAST CANCER

### A. Oophorectomy

The first report of a remission of advanced mammary cancer induced by endocrine manipulation was made by George Beatson in 1896. This was before the advent of endocrinology as a science; before the nature, and very existence, of "hormones" was understood; and long before the formulation of the concept of hormone-dependence of neoplasms by Huggins and Rous. Beatson's observations of Ayrshire cattle had led him to believe that the activities of the mammary glands and of the ovaries were, in some mysterious way, interrelated. This belief led him, empirically, to remove the ovaries of two young women with locally advanced breast cancer. He reported remission of disease in both cases. Following Beatson's pioneering reports Thomson (1902), from Edinburgh, published the results of treating 80 cases of inoperable breast cancer by means of bilateral oophorectomy. His overall response rate was 36%. A further early series of 99 cases was reported by Lett (1905). He found a response rate of 36.4%.

The operation of oophorectomy then seemed to fall into disfavour but, following the provision by Huggins and Rous of a scientific rationale for such a procedure, interest reawakened and, since 1945, numerous series have been reported in the literature. The response rates in some representative series are shown in Table 1. Regrettably, the response rate of around 36% reported by Thomson in 1902 and Lett in 1905 has not been significantly improved upon in any of the subsequent series. The data suggest that in premenopausal women response rates of around 40% can be achieved, but in postmenopausal women the response rate is, at best, around 15%. Oophorectomy has remained the "first-line" treatment of choice for the management of advanced breast cancer in premenopausal women. Over the years,

Table 1.

Response rates, complete and partial, to oophorectomy.  
(Representative series.)

SERIES	No. CASES	RESPONSE(%)
Lett, 1905	99	36
Block, 1960	60	40
Dao, 1972 (premenopausal)	259	40*
Dao, 1972 (post " )	90	15*
Veronesi, 1975	639	30*
Henderson, 1980	1674	33*

\* Indicates review of collected series.

however, a gamut of other "endocrine manipulations" has been added to the clinician's armamentarium.

#### B. Adrenalectomy

Scott and Vermeulen (1942) showed that adrenalectomy produces a profound fall in urinary sex steroids and it soon became recognised that the adrenal glands are a source, additional to the ovaries, of oestrogens (Dao, 1953). It was recognised as early as the 1940's that, by causing a reduction in circulating oestrogens, adrenalectomy might be of benefit in cases of advanced breast cancer. However, until the development of effective glucocorticoid replacement therapy (Huggins and Bergenstal, 1952) the procedure was considered too hazardous. Since the early 1950's, many series of the results of adrenalectomy have been reported. The results of some representative series are given in Table 2. The quoted response rates of 30% to 40% are similar to those achieved with oophorectomy; and adrenalectomy remains a widely practised treatment in postmenopausal or oophorectomised women with advanced disease. The procedure is of particular value in producing a further period of remission in patients who previously responded to oophorectomy (Pearson et al., 1955).

#### C. Pituitary Ablation

The beneficial effects of hypophysectomy in human breast cancer were demonstrated, independently, in Sweden and in America in the 1950's (Luft and Olivecrona, 1953; Pearson and Ray, 1959). Evidence from experimental animal work had suggested that the pituitary hormones, prolactin and growth hormone, were implicated in tumour growth. This had raised hopes that response rates to hypophysectomy would exceed those to adrenalectomy by virtue of the ablation of these additional hormones. However, the American Medical Association (1961) found that response rates to the two

Table 2.

Response rates, complete and partial, to adrenalectomy.  
(Representative series.)

SERIES	No. CASES	RESPONSE(%)
Huggins, 1952	6	33
American Medical Assoc., 1961	315	32
Dao, 1972	1384	45*
Henderson, 1980	3739	32*

\* Indicates review of collected series.



procedures were virtually identical and concluded that the effects on tumour growth of hypophysectomy are due solely to loss of ACTH. The results of representative series of pituitary ablation chosen from the literature are presented in Table 3.

#### D. Oestrogens

It is a puzzling paradox that pharmacological doses of oestrogen can produce remissions in those breast cancers thought to be "oestrogen-dependent". The mechanism of action is poorly understood but it seems possible that, in high dosage, oestrogen interferes with cytoplasmic receptor function in a manner similar to that discussed below with reference to anti-oestrogens. A second paradox is that those patients who experience a remission to oestrogen therapy may have a second remission when the hormone is withdrawn. Henderson and Canellos (1980) have collated series totalling 1683 patients treated with high dose oestrogens and report a mean response rate of 26% to the initial administration.

#### E. Anti-oestrogens

Substances which may be regarded as "anti-oestrogens" may interfere with oestrogen-target cell interaction in a variety of ways. They may simply block the access of oestrogen to its cytoplasmic receptor; they may inhibit translocation of hormone-receptor complex to the cell nucleus; or they may prevent the initiation of oestrogen-induced synthesis of DNA, RNA and protein. In hormone-dependent breast cancers, tumour growth may be regarded as an oestrogen-mediated phenomenon which can be inhibited by anti-oestrogens. Legha and Carter (1976) have suggested that this effect is brought about, not simply by competition for receptor binding, but by inhibition of the mechanism for receptor replenishment.



Table 3.

Response rates, complete and partial, to hypophysectomy.  
(Representative series.)

SERIES	No. CASES	RESPONSE (%)
Pearson, 1959	218	50
American Medical Assoc. 1961	358	31
Dao, 1972	229	44*
Henderson, 1980	1174	36*

\* Indicates review of collected series.

Three anti-oestrogens are in clinical use for the control of advanced breast cancer: tamoxifen, clomiphene and nafoxidine. Results of clinical trials of the three drugs are presented in Table 4. The response rates to all three drugs are similar to those attained with the other forms of endocrine manipulation discussed above. The most striking advantage of these drugs, particularly tamoxifen, is the almost complete absence of side effects. "Flare" and hypercalcaemia may occur, thrombocytopaenia and retinopathy have been reported, but all are rare. This class of drug must be regarded as a significant advance in the humane management of advanced breast cancer.

#### F. Aminoglutethimide

Aminoglutethimide was developed as a potential anti-convulsant but this application was abandoned when the compound's profound adrenal-suppressive effect became apparent. Its primary mode of action is to block the adrenal conversion of cholesterol to  $\Delta^5$ -pregnenolone; it thus prevents synthesis of all adrenal steroids and effectively produces what has been termed a "medical adrenalectomy". In addition, the compound is believed to inhibit peripheral synthesis of oestrogen and this action may make an important contribution to its role in the control of breast cancer (Griffiths et al., 1973). In conjunction with glucocorticoid replacement therapy, aminoglutethimide is gaining acceptance among clinicians as a valid alternative to surgical adrenalectomy in the management of advanced breast cancer. A major surgical procedure in these, often debilitated, patients is avoided and the side effects of the drug (skin rashes, drowsiness and nausea) are usually mild and transient. Henderson and Canellos (1980) have reviewed series totalling 280 cases and report response rates of between 25% and 50%. In a preliminary study from this centre, Mason et al. (1980) confirmed the ability of aminoglutethimide to suppress adrenal steroid (dehydroepiandrosterone sulphate) production and reported a response rate of 4/20 cases.

Table 4.

Response rates, complete and partial, to anti-oestrogens.  
(Representative series.)

SERIES	No. CASES	RESPONSE(%)
Legha & Carter, 1976 Clomiphene.	167	28*
Ibid. Nafoxidine.	198	31*
Ibid. Tamoxifen.	308	27*
Henderson and Canellos, 1980 Clomiphene	167	28*
Ibid. Nafoxidine.	283	31*
Ibid. Tamoxifen.	504	35*

\* Indicates review of collected series.

## G. Miscellaneous Therapies

Androgens, corticosteroids and progestogens have also been administered to cases of advanced breast cancer. The mode of action of these agents is not yet fully elucidated but corticosteroids and progestogens probably mediate their effects via adrenal and ovarian suppression respectively. Henderson and Canellos (1980) have reviewed the available data on these therapies and report mean response rates of 21% in 2250 cases treated with androgens; 25% in 508 cases treated with progestogens and 23% in 589 cases treated with corticosteroids. Prolactin antagonists have also been tried in cases of advanced disease and Dickey and Minton (1972) claim that L-dopa can produce, at least, a subjective response in some cases.

## The Present Status of Endocrine Therapy

More than 80 years have now elapsed since Beatson first demonstrated the effectiveness of endocrine ablation in some cases of advanced breast cancer. Although the number of "endocrine manipulations" available to the clinician has increased, it is regrettably apparent that the response rate of 36% achieved by Thomson and by Lett in the first decade of this century has not been improved upon. It must also be remembered that, as far as is known, no cure of advanced breast cancer has ever occurred and that the best that can be achieved with any currently available treatment is a temporary disappearance of all overt disease. More often, the remissions achieved are only partial, and may be poor compensation for the side effects of treatment. The present status of hormonal therapy for advanced breast cancer is that approximately a third of patients will obtain a remission, usually only partial, and always only temporary, from whatever form of "manipulation" is employed. This compares with an overall response rate of around 50% for currently available cytotoxic regimes (Brunner, 1978).

Clinicians have long been aware that it is inhumane to subject patients to major endocrine ablations, with all their attendant problems, on the basis of a 30% chance of, at best, a temporary remission. The dilemma of patient selection has, in part, been solved by the advent of the medical endocrine manipulations, the effects of which are, at least, reversible if therapy fails. Nevertheless, clinicians and laboratory scientists have been aware of the need for, and have energetically sought, a means of predicting which patients comprise the 30% who will respond to endocrine treatments. Such a predictor would save the remaining 70% from undergoing ineffective therapies and enable them to receive cytotoxic agents at an earlier stage in their disease, unprejudiced by the effects of prior endocrine ablation.

The potential value of an accurate index of hormone-dependence in the management of advanced breast cancer has been emphasised in the foregoing discussion. Such an index would also be applicable to the management of early disease now that the value of adjuvant systemic therapy at the time of primary local treatment is gaining acceptance. A reliable predictive index would enable the most appropriate mode of adjuvant therapy (endocrine or cytotoxic) to be selected for the individual patient.

### III. THE PREDICTION OF RESPONSE TO ENDOCRINE THERAPY IN ADVANCED BREAST CANCER

#### A. Clinical Criteria

The available data concerning the relationships between various clinical parameters and likelihood of response to hormonal therapies have been comprehensively reviewed by Fairgrieve (1965). A brief summary of his findings, together with additional data from the more recent literature, are presented here.

##### a) Site of Metastases

Fairgrieve quoted several series indicating that metastases in bone are more likely to respond to hormonal measures than those in other sites. Other workers felt, however, that response is an "all or none phenomenon" and that consideration of disease site is valueless in the prediction of response. In a more recent analysis of clinical predictors of response, Barlow and Meggitt (1968) found that none of the features examined (including disease site) was sufficiently strongly related to response to make prior selection for oophorectomy or adrenalectomy clinically feasible. Similarly, Singhakowinta et al. (1974) described a selection procedure for adrenalectomy based on clinical features and concluded that although metastases in viscera were relatively unlikely to respond, clinical indices were insufficiently precise for practical use.

Henderson and Canellos (1980) collated data from several series totalling 563 patients with metastases in bone and 203 with liver metastases. They reported a mean response rate to adrenalectomy or hypophysectomy of 38% in those with bone metastases but of only 13% in those with liver metastases. However, the response rates from the various series of cases with liver metastases ranged from



0 to 50%. These more recent data again indicate that the relationship between disease site and likelihood of response is insufficiently precise to allow exclusion of individual patients from consideration for endocrine therapy.

b) Age and Menstrual Status

Since the earliest reports, it has been recognised that the benefits of oophorectomy are largely restricted to premenopausal women. However, the relationships between menstrual status, or age, and response to the other endocrine ablations has proved more controversial. At the time of Fairgrieve's review (1965), it was thought that these factors did have a bearing on response; the evidence seeming to suggest that patients aged over 60 responded rather poorly to either adrenal or pituitary ablation, and that premenopausal women responded rather better than those past the menopause.

Henderson and Canellos (1980) concur with the view of Dao (1972) that, in general, younger premenopausal and younger postmenopausal women have a lower response rate to ablative therapies than do older women of equivalent menopausal status. They conclude, also, that for most forms of endocrine therapy, postmenopausal women respond more often than premenopausal. This last conclusion is at variance with that reached by Fairgrieve in 1965, but, being founded on an additional 15 years of clinical experience, is possibly nearer the truth.

c) Response To Previous Therapy

Much clinical evidence exists to suggest that a previous response to oophorectomy augurs well for response to further endocrine ablations. Pearson and Ray (1960) showed that 21 of 23 premenopausal women who had responded to oophorectomy and then relapsed, subsequently responded

to hypophysectomy. Conversely, 27 of 29 who had failed to respond to oophorectomy also failed to respond to hypophysectomy.

More recently, the response to pharmacological methods of endocrine manipulation has been used in selecting patients for surgical ablation. Newsome et al. (1978) treated 13 patients with aminoglutethimide for 3 months and then performed adrenalectomy on all 13. The 8 patients who had responded to aminoglutethimide also responded to adrenalectomy. The remaining 5 patients failed to respond to either treatment. The response rate of 8/13 in this small series is, of course, considerably higher than that generally reported for either aminoglutethimide or adrenalectomy. This would suggest that some element of pre-selection was operative and, perhaps, the predictive value of aminoglutethimide-response would be less striking in a more general series. A similar approach has been applied to the prediction of oophorectomy response, using medroxyprogesterone as the ovary-suppressive agent.

Information concerning response to previous hormonal therapy is, therefore, of clinical value in selecting patients for subsequent ablations. However, such information is not always available, and, should the practice of adjuvant oophorectomy become more prevalent, knowledge of response to previous oophorectomy may become a rarity. Even when available, information regarding oophorectomy response is not infallible in the prediction of subsequent response. Well-documented cases exist where response to subsequent ablative therapy has followed apparent failure to respond to oophorectomy (Kennedy et al., 1956; Cade, 1958; Randall, 1960).

#### d) Disease-Free Interval

The interval between initial treatment and the



appearance of local recurrence or metastases, the disease-free interval (DFI), is regarded as an indicator of the inherent "aggressiveness" of a particular tumour and has been evaluated as a predictor of treatment-response and of prognosis. Fairgrieve (1965) reviewed the evidence of several groups and concluded that cases with a long DFI are more likely to respond to endocrine therapies than those where the interval is short. More recently, Henderson and Canellos (1980) reviewed series totalling 2586 patients treated by adrenalectomy or hypophysectomy. They found an increasing response rate with increasing length of DFI. The mean response rate was 30% in cases with a DFI of less than 2 years and rose to 56% in cases with a DFI of greater than 5 years. Patients who initially presented with advanced disease (zero DFI) formed an intermediate group, with a response rate of 38%. This is probably because these cases were derived from 2 populations, those with very "aggressive" disease and those with slow-growing disease which they had been able to ignore for a prolonged period.

#### B. Laboratory Criteria

Clinical features, in particular DFI and the nature of response to previous therapies, can be of value in predicting response to endocrine manipulation. However, no one clinical feature, nor any "index" derived from a combination of such features, can predict, with a clinically useful degree of precision, the response of an individual patient. Response to oophorectomy might be regarded as sufficiently accurate to allow for the selection of patients for subsequent ablations, but such information is decreasingly commonly available with the increasing popularity of adjuvant oophorectomy.

A major advance in the endocrine treatment of advanced breast cancer has been the development of pharmacological agents, such as aminoglutethimide, which can be used to predict response to, or even replace, the major surgical

ablations. Nevertheless, the ability to pre-select likely responders to hormonal therapies, with a precision which cannot be achieved by consideration of clinical features alone, would be of immense value in rationalising the choice of advanced disease, and adjuvant, therapies. With these aims in view, numerous laboratory investigations have been evaluated with regard to their value as predictors of response.

a) Histology Of The Primary Tumour

Fairgrieve (1965) suggested that there was no correlation between simple aspects of tumour histology and response to endocrine therapy. More recently, Maynard et al. (1978) have compared histological grade, not directly with response to therapy, but with oestrogen receptor (E/R) status (see below) in 300 primary cancers. They found that well-differentiated tumours were more likely to possess E/R than poorly-differentiated ones, but that the difference was only significant in postmenopausal women.

Masters et al. (1978) estimated the elastica content of tumours by histochemical staining and found a correlation with E/R activity. In a subsequent report (Masters et al., 1979) they were able to correlate elastica content with response to therapy in a series of 51 patients. They reported a response rate of 50% in those where elastica was abundant in the tumour, and of only 15% in those where elastica was absent.

b) Vaginal Cytology

It may be postulated that the success of manipulations designed to control tumour growth by reducing circulating oestrogen levels requires that:

- 1) the tumour cells must have the inherent capacity to respond to oestrogen
- 2) sufficient oestrogen must be present in the body

before therapy to produce an effect on the tumour cell.

Clearly, if circulating oestrogen levels are minimal before therapy, then treatments which exert their effects by diminishing oestrogen production are doomed to failure, even if the tumour is inherently hormone-sensitive. On the basis of this simple concept the earliest laboratory attempts to select patients for endocrine ablation were designed to ascertain the "oestrogenic status" of the patient.

Cornification of vaginal epithelial cells, as observed in smear preparations, is a well-recognised index of oestrogen activity (Young et al., 1957). There is, however, no evidence to suggest that pre-operative "oestrogenic status" assessed by vaginal cytology (or by direct urinary or plasma assay) is a dominant factor in determining whether or not a particular patient will respond to ablative surgery.

#### c) Urinary Oestrogen Estimations

Methods of measuring urinary oestrogen excretion have been available since the early 1950's. Since that time groups of workers have attempted to correlate pre-treatment oestrogen excretion with response to subsequent endocrine ablation. Initially a bioassay technique was used (Bergental et al., 1955) but this was soon superseded by a more reliable and reproducible chemical method (Bulbrook et al., 1958). In their early studies Bergental et al. (1955) found that oophorectomised women with high urinary oestrogen excretion had a higher response rate to adrenalectomy than similar women whose oestrogen excretion was low. These results could not be reproduced by other workers and the assay methodology employed was much criticized.

Using the chemical method, several groups (Bulbrook et al., 1958; Swyer et al., 1961; Palmer and Helmstrom, 1962) failed to identify a significant relationship between

pre-treatment oestrogen excretion and response to subsequent adrenalectomy or hypophysectomy. It seemed, therefore, that these new chemical methods of assessing "oestrogenic status" did not fulfil their early promise, and were, in practice, no more useful than the simple expedient of vaginal cytology.

d) Estimation Of Urinary Adrenal-Steroid Metabolites

Soon after the development of the urinary oestrogen assays, techniques became available for measuring the excretion of other steroid hormones. Attempts were made to correlate the results of these estimations, in various combinations and permutations, with response to hormonal therapies. The best known, and most extensively evaluated, of these permutations was the discriminant function devised by Bulbrook et al. (1960). The discriminant function, F, was calculated from the following formula:

$$F = 80 - 80[17\text{-OH-corticosteroids (mg/24 hr)}] + \\ [etiocholanolone (\mu\text{g/24 hr})]$$

The formula was derived by consideration of the excretory levels of these steroids found in a group of women whose subsequent response to endocrine ablation was known. Positive values of "F" tended to occur in those who responded, and negative values in those who failed to respond. In the original series of patients, 13 of 14 "responders" had a positive value of "F" and 21 of 27 "non-responders" had a negative value.

Many groups of workers have attempted to confirm the predictive value of the discriminant function by calculating its value in series of patients other than those used in devising it. Unfortunately, confirmation of the value of the function could not be obtained, and further studies by Bulbrook's own group indicated that the reliability of the function was far less than had been hoped. Atkins (1968) reported on 10 years' experience with the discriminant function: in a series of 206 patients, treated by adrenalectomy or hypophysectomy, 104 had positive functions and

102, negative. The overall response rate in those with positive functions was 62% and, in those with negative values, 39%. Clearly, the number of responders among those with negative functions was too high to allow exclusion of such patients from hormonal treatment. The high overall response rate in this series suggests an element of pre-selection of cases.

e) Steroid Sulphation

Adams (1964) and Dao and Libby (1968) have shown that some mammary cancers possess enzyme systems (sulphotransferases) which enable them to synthesise sulphated esters from steroid precursors. Dao (1972) has presented data from 109 cases treated by adrenalectomy. He reported a response rate of only 15% in 46 cases with low sulphotransferase activity in the tumour, compared with 73% in 29 cases where activity was high. Of 30 cases with undetectable sulphotransferase activity, there were none who responded to treatment.

Leung et al. (1973) measured steroid sulphation in 80 patients with breast cancer. They related the capacity of the tumour for sulphation to E/R activity (see below) and found a reasonable correlation. However, in 22 cases of advanced breast cancer, they found no correlation between sulphotransferase activity and response to endocrine ablation.

f) Sex Hormone Binding Globulin (SHBG)

Murayama et al. (1979) measured SHBG levels in the peripheral blood of 109 women with breast cancer. They reported a correlation between SHBG level and E/R activity (see below). They further reported, that in 50 cases treated by endocrine measures, 20 of 22 with high SHBG levels responded, compared with only 5 of 28 with low SHBG levels.

These findings are yet to be reproduced in other centres. If they are confirmed, then a predictive test which can be performed on a peripheral blood sample would offer a significant advantage over the tests, to be described below, which require a tumour biopsy. Preliminary results from this centre indicate, however, that there is no direct relationship between plasma SHBG levels and tumour E/R content (Mason et al., 1980).



#### IV. OESTROGEN RECEPTOR STATUS

In 1959, Glascock and Hoekstra showed that "oestrogen-target" tissues, such as uterus and vagina, have a greater capacity to "take up" oestrogen than do "non-target" tissues such as kidney or liver. They demonstrated this by injecting tritium-labelled hexoestrol into goats or sheep and, after varying time intervals, measuring the radioactivity taken up by "target" and "non-target" tissues. They found a marked, selective localisation of tritium in the "oestrogen-target" tissues. Jensen and Jacobson (1960) were able to demonstrate a similar "selective uptake" in studies using tritiated oestradiol in laboratory animals. Through these very elegant studies and his subsequent work, the name of Elwood Jensen has, rightly, become legendary in the receptor field.

Following this pioneering work, the concept evolved that this selective uptake of oestrogen is due to the presence, in "oestrogen-target" tissues, of intracellular macromolecules with a high affinity for oestrogen. These macromolecules have become known as oestrogen receptors (E/R). Soon after these demonstrations that physiological target tissues for oestrogen action in experimental animals exhibit a high affinity for oestrogen, it was postulated that oestrogen-dependent breast cancers might also possess such a property. Folca et al. (1961) injected tritiated hexoestrol into 10 patients with advanced breast cancer prior to surgical endocrine ablation. Portions of tumour and of skeletal muscle were then removed at surgery and the ratio of tritium-labelling between the two tissues determined. In 4 patients, tritium-labelling of tumour far exceeded that of muscle, and all 4 cases subsequently responded to ablation. In the remaining 6 patients, the difference in labelling between tumour and muscle was much less marked and all 6 failed to respond to ablation.

Following this in-vivo demonstration that the oestrogen binding capacity of a tumour seems to predict its response to endocrine ablation, much energy has been

directed towards the development of techniques for measuring, in-vitro, the oestrogen-binding capacity, or E/R content, of breast cancer biopsy specimens.

#### A. Techniques Used For E/R Estimations

Most methods for estimating E/R activity in tumour biopsy specimens employ the same basic principle. A portion of tumour (around 300 mg in our laboratory) is homogenised in buffer and centrifuged to yield a tissue extract, the "cytosol", which contains the E/R, a soluble protein. A portion of this cytosol is then incubated with radioactive oestrogen to fill empty E/R sites, i.e. those not already occupied by endogenous hormone. The radiohormone-protein complex ("bound hormone") is separated from the unbound, excess radiohormone by one of several methods and the amount of radioactivity in the "bound" fraction measured by scintillation counting.

Most of this "bound" oestrogen will be attached to E/R, but some will be attached to low affinity, non-specific binding sites. A measure of this non-specific binding can be obtained by a refinement of the assay procedure. An additional portion of tumour cytosol is incubated with a large excess of non-radio-active oestrogen to "saturate" the available E/R. Any residual ability of the cytosol to bind radiohormone is attributable to non-specific binding which, although of low affinity, is unsaturable.

A brief summary of the various techniques for E/R assay which are in current use follows. The techniques differ mainly with respect to the method used for separating "bound" from "free" hormone after incubation with the cytosol.

##### a) Dextran-Coated Charcoal (DCC)

A suspension of dextran-coated charcoal (DCC) is



added to the cytosol after incubation with hormone. "Free" hormone is adsorbed to the charcoal which is then removed by centrifugation. This method was first described by Korenman and Dukes (1970) and is the basis of the technique used in many laboratories. The method used in the experimental work described in this thesis (Hawkins et al., 1977) is based on this approach.

The currently used technique involves the incubation of multiple aliquots of cytosol with a fixed mass of radio-hormone and varying masses of unlabelled hormone. Data regarding the amount of radiohormone in the "bound" fraction are obtained for each aliquot and analysed by the method of Scatchard (1949), thus yielding a value for the concentration of E/R (Po) and for the dissociation constant of binding (Kd). This latter parameter helps to ensure distinction between specific and nonspecific binding. The application of Scatchard analysis to E/R data has been discussed in detail by Chamness and McGuire (1975) and by Braunsberg and Hammond (1979).

#### b) Sucrose Density Gradient (SDG)

This technique is also widely used for the separation of "Bound", from "Free", hormone. Two aliquots of cytosol are incubated; one with a single, saturating dose of radio-hormone, the other with radiohormone plus a vast excess of unlabelled hormone - for the estimation of non-specific binding. After incubation, the cytosols are loaded into tubes containing SDG's and centrifuged, either overnight, using a "swing-out" rotor, or for 3 hours, using a vertical tube rotor. By this means, molecules are distributed throughout the SDG, mainly according to their molecular weights; large molecules accumulating at the bottom, and small ones at the top of the gradient. Oestrogen - E/R complex accumulates, therefore, towards the bottom of the SDG and "free" hormone towards the top. Fractions are collected from the gradients into vials of scintillator and the amount of radioactivity in

each fraction is measured. The amount of E/R in the cytosol can be derived from the amounts of radioactivity in the high molecular weight ("bound") fractions of the "total" and "non-specific" binding tubes.

E/R is found to exist in at least two molecular forms, sedimenting in the "4S" and "8S" regions of the gradient.\* One advantage of the SDG, rather than the DCC, approach is that it enables the "4S" and "8S" forms to be quantitated separately. This separation is useful in the research field, and, as discussed below, may be of clinical significance. The SDG technique is used in many contemporary laboratories, notably that of Jensen and his co-workers (Jensen et al., 1975). SDG analysis was the research tool used in establishing the molecular characteristics of the E/R protein (Toft and Gorski, 1966).

#### c) Miscellaneous Separation Techniques

Other, less widely used, techniques include precipitation of the "bound" hormone with protamine sulphate (Steggles and King, 1970); gel filtration (Godefroi and Brooks, 1973) and electrophoresis (Teulings et al., 1975).

#### B. Clinical Value of E/R Status

The incidence of "E/R-positivity" varies between centres and between methods of assay. Hawkins et al., (1980) have reviewed this subject and report that 59 - 72% of tumours are designated E/R-positive by DCC analysis. Numerous reports have been published concerning the correlation between E/R status and response to various forms of endocrine manipulation. Major reviews on this subject include those by Henderson and Canellos (1980) and Hawkins et al. (1980). Some representative figures are presented in Table 5. Overall, about 40 - 60% of E/R-positive tumours respond to ablation or hormone administration, compared with

\*Where "S" refers to the Svedberg unit, a measure of sedimentation constant.

Table 5.

E/R-status and response to endocrine therapies.

(Representative series.)

THERAPY.	AUTHORS.	No. CASES.	Response. (E/R-pos.)	%	Response. (E/R-neg.)	%
Oophorectomy	Hawkins et.al.	225	53/91	58	7/134	5
"	Henderson &Canellos.	90	25/33	76	4/53	7
Adrenalectomy	Hawkins et.al.	62	13/33	39	0/29	0
"	Henderson &Canellos.	99	32/66	48	4/33	12
Oestrogens	Hawkins et.al.	169	65/105	62	4/64	6
"	Henderson &Canellos.	115	37/57	65	5/58	9
Anti- oestrogens	Hawkins et.al.	235	74/138	54	14/97	14
"	Henderson &Canellos.	156	59/116	59	2/40	5

well under 10% of E/R-negative tumours. One exception to this generalisation is that Hawkins et al. (1980), in a review of series totalling 235 cases treated with anti-oestrogens, found a response rate of 14% in 97 cases designated E/R-negative. However, in their review of 156 cases treated with tamoxifen, Henderson and Canellos found a response rate of only 5% in 40 cases designated E/R-negative.

Knowledge of E/R status, as assessed by current techniques, enables the clinician to spare about 30% of patients with advanced breast cancer from undergoing valueless endocrine therapy. Very few (less than 10%) of these, the E/R-negative cases, would have responded had they been so treated. Viewed in another way, E/R status enables the most appropriate mode of treatment for advanced disease to be selected in over 65% of cases: the 30% who are E/R-negative are identified for treatment with chemotherapy, the 35% who respond to endocrine measures are allocated to this form of treatment by virtue of their E/R-positive status and only 35% of cases, the E/R-positive but non-hormone responsive group receive "sub-optimal" therapy. Thus, the "predictive value" of negative E/R status exceeds 90%, but of positive E/R status, is only 40 - 60%.

### C. Limitations of E/R Status

E/R status is clearly a significant advance over clinical criteria and previously evaluated laboratory parameters in terms of its predictive value. However, E/R remains far from ideal as a clinical tool. Firstly, conventional methodology, either DCC or SDG, is cumbersome, requiring the use of some 300 mg of tumour. Such a quantity is often unavailable in patients presenting with a small primary tumour or with relatively inaccessible metastases, such as those in bone or liver. For these patients, E/R status cannot readily be determined to assist in the selection of appropriate therapy. Secondly, these current techniques

require the use of radioactive materials and expensive equipment for homogenisation, centrifugation and scintillation counting. Such techniques are restricted, therefore, to special centres and are unsuitable for adoption by peripheral hospitals. Thirdly, despite its advantages over previous predictors of response, E/R status remains an imperfect predictor for the individual patient, in that only about 50% of E/R-positive tumours are endocrine-responsive. Fourthly, the biochemical technique obscures the heterogeneity of breast tumours by "pooling" the cytosol from epithelial cells and stroma and, possibly, from E/R-positive and negative tumour cell populations.

Recently much research has been directed at overcoming these limitations of E/R status. On the one hand, attempts are being made to "stream-line" the techniques for identification of E/R so that they may be performed with less expense, and on smaller specimens. On the other hand, independent parameters continue to be evaluated in an attempt to identify the subgroup of E/R-positive tumours which will actually respond to endocrine manipulation. These two approaches to overcoming the limitations of E/R status will now be discussed.

#### D. Recent Advances In Techniques For The Identification Of E/R

##### a) Conventional Assays On Smaller Samples

Several groups of workers are developing techniques which require a much smaller mass of tumour than the 300 mg preferred in our laboratory, but which still involve the same steps of cytosol preparation, incubation with radio-hormone and separation of "bound", from "free", hormone. Such approaches include an assay based on iso electric focusing (Wrang et al., 1979) and a variant of the DCC technique (Poulsen et al., 1979). The latter group have



attempted to determine E/R status in cytosols prepared from fine-needle aspirates of tumours. They admit, however, that the "false-negative rate" is unacceptably high, due, probably, to the low cellularity of some aspirates.

b) Immunohistological Techniques

An alternative to the development of biochemical techniques "in miniature" as discussed above, is the application of immunohistological techniques to the identification of E/R in histological sections of tumour. Work is progressing in several centres towards raising an antibody to the E/R protein. As yet, however, an antibody which will bind to human tumour E/R is unavailable. All current immunohistological techniques for the localisation of E/R depend, therefore, on the use of a labelled antibody to oestradiol, and thence to an indirect assessment of the presence of E/R in the tissue.

Pertschuk et al. (1978) described one such technique in which frozen sections of breast cancers were incubated with polymerized oestradiol which became bound to any E/R present. The sections were subsequently exposed to sheep, anti-oestradiol serum and, finally, to fluorescein-conjugated, rabbit, anti-sheep serum. This system permitted the localisation of bound oestradiol when the sections were examined by fluorescence microscopy. Control sections, processed to identify any non-specific fluorescent staining, were incorporated in the technique. A 95% correlation with results of DCC and SDG techniques was reported using this system.

Walker et al. (1980) reported briefly on a similar technique. They incubated frozen sections with non-polymerised oestradiol and subsequently exposed the sections to rabbit, anti-oestradiol serum, followed by anti-rabbit gamma-globulin raised in swine and, finally, to peroxidase-antiperoxidase complex. This system permitted indirect localisation of

E/R under the light microscope after staining for peroxidase using diaminobenzidine. These workers found the number of positively-staining tumours to be very small. Mercer (1979) has evaluated both immunofluorescent and immunoperoxidase techniques for the localisation of E/R. Like Walker et al., he used non-polymerised oestradiol for the initial binding reaction to E/R. An 85% correlation with the results of DCC assay is reported using these systems.

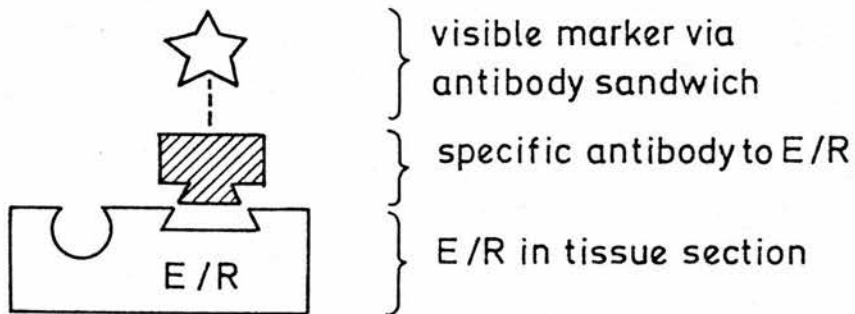
Kurzon and Sternberger (1978) have described an immunoperoxidase technique for the identification of E/R in fixed, rather than frozen, sections. Their method involves incubation of tissues with oestradiol prior to fixation to "protect" the receptor. Subsequently, sections are exposed to anti-oestradiol serum. The technique is not applicable, therefore, to the examination of specimens which have been fixed and stored in the usual way. The principles of the immunohistological techniques are illustrated in Figure 1.

#### c) Histochemical Techniques

Despite the apparently excellent agreement of the results of their immunohistological technique with those of conventional assays, Pertschuk et al. (1979) published an "improved" method for the detection of E/R in frozen sections. This method involved incubation of sections with a conjugate of oestradiol, albumin and fluorescein. Controls to identify non-specific fluorescent-labelling, included equivalent sections co-incubated with an excess of unconjugated oestradiol, or analogue; and sections incubated with a conjugate of albumin and fluorescein only. A 92% agreement with the results of DCC assay was reported using this system. The authors stated the advantages of this method over their previous immunohistological system to be the better availability and reproducibility of the reagents.

Lee (1978) has also examined frozen sections for E/R

## 'IDEAL' SYSTEM



## AVAILABLE SYSTEMS

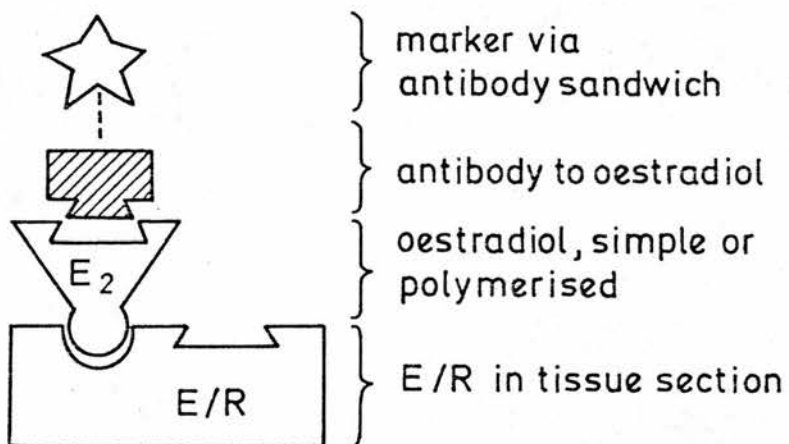


Figure 1.

Diagrammatic representations of possible immunohistological approaches to E/R identification.



using a similar conjugate of oestradiol, albumin and fluorescein. Of 17 tumours examined he found histochemical evidence of E/R in 15.

Walker et al. (1980) have also evaluated a histochemical technique. They used a conjugate of oestradiol, albumin and peroxidase, rather than fluorescein. A good correlation, in qualitative terms, was reported between the results of histochemistry and DCC assay. The principles of the histochemical techniques are illustrated in Figure 2.

The experimental work to be described in this thesis includes an evaluation of histochemical techniques based on those of Pertschuk et al. (1979) and of Walker et al. (1980).

## E. Recent Alternatives To E/R Status In The Prediction Of Response To Endocrine Therapy

### a) Receptor Quantitation

In current clinical practice, E/R assays are used to assign a tumour to either the E/R-positive or E/R-negative category. Treatment decisions are then based on this simple classification. It has been suggested that a more precise consideration of the amount of E/R in the cancer cell might improve prediction. McGuire et al. (1978) reported on over 100 patients in whose breast cancer metastases E/R had been assayed quantitatively. They divided the tumours into 3 categories on the basis of increasing E/R content and found response rates to endocrine therapy of 6%, 46% and 81% in successive categories. Jensen (1975) and Leclercq and Heuson (1977) report a similar relationship between likelihood of response and concentration of E/R. However, Hawkins et al. (1979) found that such a relationship applied only to premenopausal women.

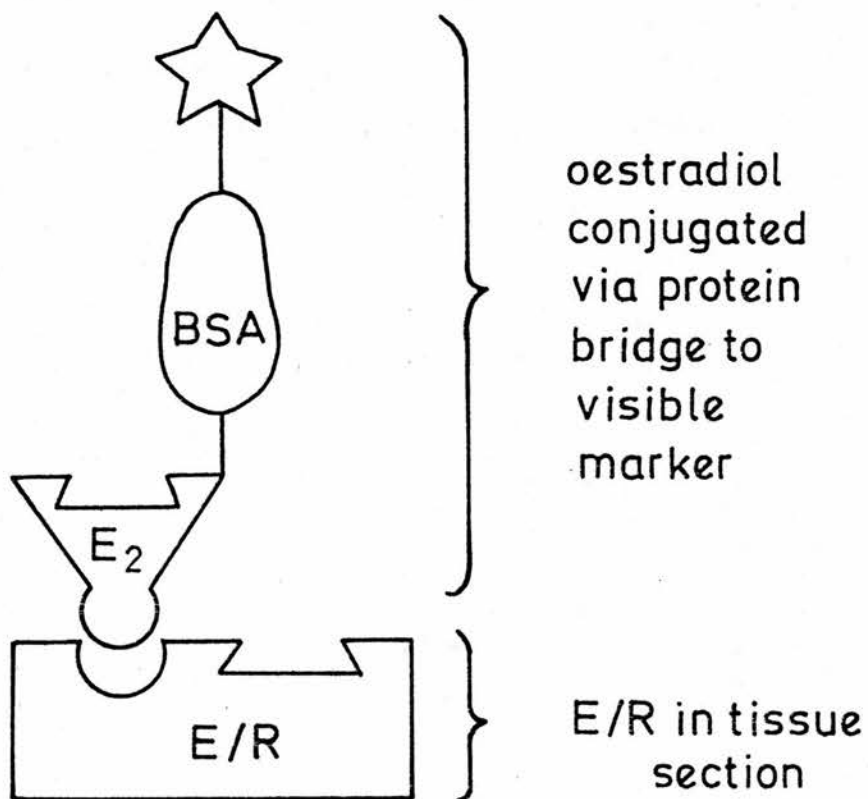


Figure 2.

Diagrammatic representation of histochemical approach to E/R identification.

b) Nuclear E/R Assay

It has been postulated that, since E/R must be translocated to the cell nucleus in order to mediate tumour growth, identification of E/R within the nucleus might provide a better indication of hormone-dependence than that afforded by identification of E/R within the cell cytoplasm. Several reports (Anderson et al., 1972; Kiang, 1977; Laing et al., 1977; Leake, 1977; Panko and MacLeod, 1978; Zava and McGuire, 1977) have described the preparation of nuclear pellets from breast cancers and the assay of these for E/R. Leake has assayed both cytoplasmic and nuclear E/R in 250 breast cancers; he found that 30% possessed both. Data concerning response to endocrine therapy was available for 43 of the cases. Twenty of 27 cases with both cytoplasmic and nuclear E/R responded; compared with only 1 of 6 with cytoplasmic E/R alone and 1 of 8 with neither. These data are interesting but are clearly too preliminary to allow conclusions about the clinical value of nuclear E/R assay to be drawn.

c) Molecular Form Of E/R

By means of SDG assay, at least 2 molecular forms of cytoplasmic E/R, ("4S" and "8S"), can be distinguished. Savlov et al. (1977) have suggested that possession of the "8S" form is a prerequisite for response to hormone manipulation, tumours possessing only "4S" receptor being unlikely to respond. The usefulness of this distinction has not been confirmed by studies carried out in this department (Freedman and Hawkins, 1980).

d) Progestogen Receptor

A cytoplasmic receptor for progestogens (Pg/R) can be identified in oestrogen-dependent tissues, such as

uterus, by techniques analogous to those used to identify E/R. Horwitz et al. (1975) postulated that the synthesis of Pg/R in such tissues is oestrogen-dependent, requiring that E/R be bound to oestrogen, and be "translocated" to the cell nucleus; i.e. requiring the presence of a functional E/R. They suggested, therefore, that Pg/R, as an index of functional E/R, might be a better index of hormone-dependence than the mere presence of cytoplasmic E/R.

McGuire et al. (1978) reported on 71 patients in whose breast cancer metastases both E/R and Pg/R had been measured. A response rate to hormonal therapies of 83% was found in cases possessing both E/R and Pg/R, compared with only 29% in cases possessing E/R alone. These figures suggest that the response rate in Pg/R-negative cases is too high to permit patient selection on this basis. The experimental work described in this thesis includes an evaluation of Pg/R as an indicator of hormone-dependence in rat mammary tumours.

#### e) Androgen Receptor

Androgen receptors (A/R) have been less widely investigated, with reference to breast cancer, than have E/R and Pg/R. Persijn et al. (1975) measured A/R in 51 breast cancers. They found that 4 of 6 patients with A/R in their tumours responded to oophorectomy compared with none of 3 patients without A/R. They found, however, that A/R status had no predictive value for response to additive hormone therapy (ethinyl oestradiol), whereas E/R exhibited a predictive value for both ablative and additive treatments. Studies on A/R in breast tumours are in progress in our own laboratories.

## V. ENDOGENOUS PEROXIDASE

It is postulated that when oestrogen enters a target cell, it becomes bound to E/R, is translocated to the nucleus and activates the genetic material to synthesise proteins such as Pg/R and further E/R. Another protein whose synthesis is thought to be induced by oestrogen action in this manner is an enzyme, peroxidase. The evidence, from the literature, which indicates that peroxidase might serve as a marker for a "functional E/R", and which prompted much of the experimental work described in this thesis, is reviewed below.

### A. Peroxidase As A Marker For A "Functional E/R"

Lucas et al. (1955) reported that the administration of diethyl stilboestrol or oestradiol-17 $\beta$  to oophorectomised rats produced a pronounced peroxidase activity in their uteri. However, these workers did not view the peroxidase as a marker for oestrogen-dependence, simply suggesting that it might serve as a marker for "tissue proliferation". These early studies indicated that the oestrogen-induced peroxidase activity might be wholly attributable to an influx of peroxidase-rich eosinophils. In 1969, however, Brockelmann and Fawcett were able to demonstrate, by electron microscopy, that peroxidase activity is induced within uterine epithelial cells.

The induction of peroxidase in rat uteri by oestrogens, and also by anti-oestrogens, was further investigated by Churg and Anderson (1974), Anderson et al. (1975), Jellinck et al. (1976) and Jellinck and Newcombe (1977). These later studies indicated that the peroxidase is truly a marker for oestrogen action, not simply for tissue proliferation, nor for a non-specific effect of steroid hormones in general. Jellinck and Newcombe (1977) were able to demonstrate a

close relationship between the uterotrophic and peroxidase-inducing effects of a number of steroids and proposed that peroxidase assay might be useful as a technique for the determination of oestrogenic activity of compounds.

Peroxidase was measured in mammary tumours as long ago as 1955, when the enzyme was regarded only as a marker for tissue proliferation. In that year, Neufeld et al. were able to demonstrate peroxidase activity in the mitochondria of Walker 256 rat mammary tumour models. The possibility that peroxidase might serve as a useful marker for hormone-dependence in mammary tumours was first raised by Anderson et al. (1975). By means of a histochemical technique, they showed peroxidase activity to be present in alveolar cells of growing DMBA-induced tumours, but to be absent from such tumours regressing after oophorectomy. A further report from the same group (DeSombre et al., 1975) confirmed these findings in a small number of DMBA-induced tumours, and, in addition, indicated that peroxidase was absent from actively growing but ovary-independent rat mammary tumour models.

A further study in animal tumour models of hormone-dependent and -independent growth was reported by Lyttle et al. (1979). They measured peroxidase in a total of 45 mouse mammary tumours. They found a highly significant difference in peroxidase levels between the hormone-dependent and -independent groups, with no "overlap" between the ranges of values. This suggested that, at least in these experimental models, peroxidase serves as an ideal marker for hormone-dependence.

Peroxidase activity was measured in 39 human tumours by Lyttle and DeSombre (1977). They demonstrated a wide spectrum of activities, 9 tumours having no detectable peroxidase, and some having very high levels - approaching those found in uterine specimens. They did not correlate these findings with either response to endocrine therapy or



with E/R status. Duffy and Duffy (1977) correlated peroxidase activity with E/R status in a series of 52 human cancers of those designated E/R-positive, 78% had demonstrable peroxidase, compared with only 20% of those designated E/R-negative.

#### B. Molecular Characteristics Of Oestrogen-Induced Peroxidase

The oestrogen-induced, endogenous peroxidase of oestrogen-target tissues (E.C. 1.11.1.7.) is thought to be a carbohydrate - containing haem protein, like other mammalian peroxidases (DeSombre and Lyttle, 1978). The same group have estimated the molecular weight of mammary tumour peroxidase by gel filtration. They obtained a value of 50,000, similar to the M.W. of uterine peroxidase but significantly less than that of other mammalian peroxidases.

Many groups have undertaken electron microscope studies to determine the subcellular localisation of the enzyme. In their studies on rat uterus, Brockelmann and Fawcett (1969) reported the enzyme to be present in the rough endoplasmic reticulum and nuclear envelope of epithelial cells, but to be absent from the Golgi apparatus. Churg and Anderson (1974) and Anderson et al. (1975) reported a similar distribution of the enzyme in uterine epithelial cells, but found that it was also present in the Golgi. In studies of DMBA-induced rat mammary tumours, (Anderson et al., 1975 and DeSombre et al., 1975) and of healthy, lactating rat mammary gland (Anderson et al., 1975) a similar subcellular distribution of enzyme was found and also a strongly positive peroxidase-reaction in the lumens of ducts was noted, suggesting secretion of enzyme.

Investigation of the time-course of oestrogen-induced, peroxidase synthesis indicates that the enzyme begins to appear 4 hours after oestrogen administration, is maximal at 20 hours and declines thereafter, (Lyttle and DeSombre, 1977). Earlier studies had suggested that the enzyme did





not begin to appear until 12 hours after oestrogen-stimulation (Brockelmann and Fawcett, 1969) and that its activity was maximal at 72 hours (Lucas et al., 1955). The half-life of the oestrogen-induced peroxidase is reported to be around 4 hours (Jellinck et al., 1976).

### C. The Physiological Role Of Oestrogen-Induced Peroxidase

The experimental work described above has indicated that synthesis of peroxidase is induced, in target tissues, by oestrogen action; and that this enzyme can be "exploited" as a useful marker for oestrogen action. Groups of workers investigating this oestrogen-induced peroxidase have postulated various possible physiological functions for the enzyme and have attempted to elucidate its biological role.

One theory is that a peroxidase system potentiates the conversion of oestrogens to phenoxy radicals and their binding to proteins, (Williams - Ashman & Johnson, 1960; Williams - Ashman, 1965; Brockelmann, 1969; Jellinck & Fletcher, 1970; Williams - Ashman & Reddi, 1971). In this way, the presence of peroxidase, induced by an "oestrogen-to-receptor-interaction", might serve to catalyse further binding of oestrogen to its receptor.

Other workers (Klebanoff & Segal, 1960; Jellinck & Irwin, 1963; Lyttle & Jellinck, 1972; Jellinck & Lyttle, 1972; Jellinck & Lyttle, 1973) suggest that a peroxidase system is involved in the conversion of oestrogens to inactive, water-soluble metabolites, thus limiting their duration of action on the target tissue. The peroxidase-catalysed conversion of oestrogen to water soluble products is well documented, and is the basis of an established assay for peroxidase activity (Jellinck & Newcombe, 1977).

The peroxidases of various body fluids (e.g. saliva, milk and tears) have been shown to exhibit bactericidal and virucidal properties (Klebanoff and Luebke, 1965; Klebanoff et al., 1966; Belding et al., 1970). It may be

that in the genital tract, also, such a function is of importance. A final theory is that peroxidase may be responsible for the "uterine-fluid mediated, sperm-inhibitory system" described by Klebanoff and Smith (1970) and Smith and Klebanoff (1970).

#### D. Techniques For The Assay Of Endogenous Peroxidase Activity

Endogenous peroxidase catalyses reactions of the general form:



where  $\text{DH}_2$  represents a hydrogen donor. The reaction can be regarded as one with 2 substrates, the hydrogen donor and the peroxide. The enzyme exhibits a high degree of substrate specificity with regard to the peroxide, only hydrogen, acetyl, methyl or ethyl peroxides serving as substrates. With regard to the hydrogen donor, however, the enzyme exhibits broad specificity. Many hydrogen donors which give rise to coloured oxidation products can serve as substrates, and these can conveniently be used for the assay of peroxidase activity, as the rate of appearance of the coloured products can be measured by spectrophotometry.

Many hydrogen donors have been described for use in biochemical assay systems (Maehly and Chance, 1954; Putter, 1974). Putter recommends di-o-anisidine as producing the most accurate results; however, this compound is carcinogenic and guaiacol is, therefore, recommended for most uses. The first description of the guaiacol reaction for the assessment of peroxidase activity was made by Lepinois (1899) and most biochemical studies of oestrogen-induced peroxidase have employed a guaiacol method based on that of Himmelhoch et al. (1969). Herzog and Fahimi (1973) described a colourimetric assay method based on 3,3'-diaminobenzidine as hydrogen donor and claimed a better sensitivity than can

be achieved with the guaiacol methods. A very different approach to the assay of peroxidase activity was used by Jellinck and Newcombe (1977). The ability of peroxidase to accelerate the conversion of ( $^{14}\text{C}$ ) oestradiol to water-soluble products provided the basis for their system.

A guaiacol method was chosen for the studies described in this thesis because of its long-established use, and the fact that it is the method of choice of other groups working in closely related fields (Lyttle and DeSombre, 1977; Duffy and Duffy, 1977; DeSombre and Lyttle, 1978; Lyttle et al., 1979; Collings and Savage, 1979).

Although the guaiacol reaction is long-established for the assay of peroxidases, the chemical structure of the oxidation product is poorly understood. Booth and Saunders (1956) reported that, probably, more than one compound results, and that a definite stoichiometric formula cannot be given. The product, generally known as "guaiacol dehydrogenation product" has a characteristic orange/brown colour with an absorbance peak at 470 nm. Measurement of the increase in absorbance at this wavelength by means of spectrophotometry enables the peroxidase activity in solutions under study to be deduced.

Unlike E/R and Pg/R, endogenous peroxidase is not a component of the soluble cytoplasm of cells, being associated with the endoplasmic reticulum and nuclear membrane. Because of the insoluble nature of the enzyme, peroxidase activity is minimal in the cytosols prepared for E/R and Pg/R assay. To produce a tissue extract suitable for biochemical assay of peroxidase, the enzyme activity can be solubilised by the addition of calcium chloride to the homogenisation buffer. DeSombre and Lyttle (1978) have shown that a concentration of 0.5 M calcium chloride produces optimal peroxidase activity in tissue extracts. Higher concentrations reduce the specific activity of the solubilised enzyme, due to increased extraction of other, "interfering" proteins.

A histochemical technique for the localisation of peroxidase has also been evaluated in this work. The method chosen measures the ability of endogenous peroxidase in a frozen section of tissue to catalyse the oxidation of diaminobenzidine (DAB) to yield a brown reaction product, visible at the light microscope level. The product in this reaction is an insoluble, phenazine polymer.

VI. CURRENT STATUS OF THE PREDICTION OF RESPONSE TO  
ENDOCRINE THERAPY

In this introductory review, I have attempted to justify the search for a predictor of response to endocrine therapies: both for the rational, palliative treatment of advanced breast cancer, and for the selection of adjuvant therapy in cases of early disease. The historical review of attempts to predict response brings me to conclude that, at the present time, E/R status, as assigned by DCC or SDG assay, provides the most precise prediction available. The main limitations of E/R status, as a clinical tool, are related, firstly to the fact that many E/R-positive tumours fail, in practice, to respond to hormonal measures and so a more precise predictor must be sought; and, secondly, to the fact that the assay methodology is inconveniently complex, cumbersome and expensive, and thus considered unsuitable for routine hospital use.

The experimental work described in this thesis has been aimed at overcoming these 2 limitations of E/R status. Firstly, a postulated alternative predictor of response, endogenous peroxidase, has been evaluated in the hope that it might provide more accurate prediction. Secondly, histochemical approaches to E/R identification have been evaluated in the hope of devising a simple system, more suited to routine hospital use than current techniques.

PART IIEXPERIMENTAL METHODS

# 1. ASSAY OF OESTROGEN RECEPTOR (E/R) ACTIVITY IN TISSUE CYTOSOLS

These assays were performed by the method of Hawkins et al. (1975), incorporating the modifications described by Hawkins et al. (1977). The method is based on the original work of Korenman (1968) and Feherty et al. (1970).

## REAGENTS

1. Tris Buffer (0.25M Sucrose; 10mM Tris; 1mM EDTA; pH 8.0).
2. Tris - glycerol - monothioglycerol buffer (10% glycerol and 1% monothioglycerol in Tris buffer).
3. (2,4,6,7 -  $^3\text{H}$ )oestradiol 17 $\beta$  (10 pg/100ul in Tris) from New England Nuclear Corporation. ( [  $^3\text{H}$  ]  $\text{E}_2$  )
4. Oestradiol 17 $\beta$  from Sigma. ( $^0\text{E}_2$ )
5. Dextran/charcoal suspension: 1.5g/l of Norit A charcoal (Sigma) in dextran, 0.015g/l in Tris.
6. Organic scintillator solution: 21 ml Liquifluor (New England Nuclear Corporation), 10 ml Ethanol, 490 ml toluene.

## METHOD

Rat tissues were dissected free from the host animals, following exsanguination, and kept on ice. Human mammary tumours were collected, on ice, direct from the operating theatre. After weighing, tissues were homogenised, on ice, in the Tris - glycerol - monothioglycerol buffer at a concentration of 300 mg/ml (rat tissues) or 100 mg/ml (human tissues) in a plastic tube (Luckham PT/1772) using a



Silverson homogeniser for 3 x 15 sec. episodes. The resulting homogenates were centrifuged at 25,000 rpm for 45 min. in an MSE Superspeed 50 centrifuge (rat tissues) or at 3,200 rpm for 20 min. in an MSE Mistral 6L centrifuge (human tumours). The supernatant (cytosol) was then removed with a Pasteur pipette for E/R and Pg/R assay.

This procedure was modified in the case of tissues from which the centrifugation pellets were subsequently to be processed for assay of endogenous peroxidase. Monothioglycerol inactivates peroxidase (DeSombre et al., 1978), and so was omitted from the homogenisation buffer and added to the cytosols (at approximately 1%) after separation from the pellets.

Assay tubes for E/R saturation analysis were prepared, in duplicate. Each tube contained 10 pg of [ $^3\text{H}$ ]  $\text{E}_2$  and a variable mass of  $^0\text{E}_2$  (0, 10, 30, 50, 70 and 90 pg). The volume in each tube was made up to 1 ml with Tris buffer. For the estimation of "non-specific binding", tubes were prepared containing 1,000 and 20,000 pg of  $^0\text{E}_2$ .

After mixing of the tubes, 100  $\mu\text{l}$  of the tissue cytosol under study was added to each. After 30 - 45 min., 10 pg of [ $^3\text{H}$ ]  $\text{E}_2$  was added to each of the "non-specific binding" tubes, giving sufficient time for the  $^0\text{E}_2$  to "saturate" any E/R in the cytosol. After further mixing, all tubes were left to incubate, overnight, at  $4^\circ\text{C}$ .

The next morning, 0.5 ml of charcoal suspension was added to each tube, the contents mixed, allowed to stand for 15 min. at  $4^\circ\text{C}$  and then centrifuged at 2,500 rpm for 10 min. in the MSE Mistral 6L centrifuge. The supernatants ("bound" fractions) were then decanted into plastic vials, each containing 5 ml of organic scintillator. The vials were heated for 2 hr. to promote disruption of steroid-receptor complex, and transfer of steroid to the organic phase of the counting system. All vials were then subjected to scintillation counting for sufficient time to reduce the

coefficient of variation of counting to less than 5%.

In addition, duplicate, standard tubes containing 10 pg of [ $^3\text{H}$ ]  $\text{E}_2$  in 1.5 ml Tris buffer were processed with each batch of assay tubes. The contents of these tubes were transferred directly to counting vials as a measure of the total amount of [ $^3\text{H}$ ]  $\text{E}_2$  available for E/R binding.

The presence or absence of E/R in the cytosol under study was then determined by inspection of the raw data. In cases where E/R was seen to be present, the data were plotted according to the method of Scatchard (1949) to yield values for the concentration of E/R present ( $\text{P}_0$ ) and the dissociation constant of binding ( $\text{K}_d$ ). E/R concentrations were expressed in fmoles per mg of wet tissue.

## 2. ASSAY OF E/R ACTIVITY IN A SOLID - PHASE SYSTEM

Studies into the effects of various forms of "fixation" or "protein - immobilisation" on E/R activity required a system for the assay of E/R in a "solid phase". Such assays were performed by a method based on that of Lippman and Huff (1976), derived from the original work of Steggles and King (1970) and Chamness et al. (1975). Lippman and Huff used their method to measure E/R activity in protamine - sulphate - precipitated pellets. In the present work, pellets derived from precipitation with both protamine - sulphate and acetone were examined.

For the protamine - sulphate assay, cytosol was prepared as for DCC assay and a 200  $\mu$ l aliquot was pipetted into each of 7 glass tubes. To each tube was added 200  $\mu$ l of a 1.5 mg/ml solution of protamine sulphate (Sigma) in Tris buffer. The tubes were mixed, allowed to stand at 0°C for 5 min., centrifuged at 3,200 rpm for 10 min. and the supernatants removed by suction. The pellets containing the precipitated E/R activity were then incubated with 10 pg of [ $^3$ H] E<sub>2</sub> and varying masses of  $^0$ E<sub>2</sub> (0, 10, 30, 50, 70, 90, 1000 pg) in a final volume of 200  $\mu$ l of Tris buffer. After incubation at 4°C, overnight, the incubation-mixture was removed by suction and the pellets washed with 3 x 2 ml of buffer. The pellets, containing any "bound" [ $^3$ H] E<sub>2</sub>, were then dissolved in 0.5 ml of ethanol and transferred to vials of scintillator fluid. The amount of [ $^3$ H] E<sub>2</sub> bound to each pellet was determined by scintillation counting and the data analysed by the method of Scatchard as described for DCC assay. Each assay was performed in duplicate.

### 3. ASSAY OF E/R ACTIVITY IN GLASS-MOUNTED, FROZEN SECTIONS

This technique was used during preliminary investigations to establish the feasibility of a histochemical approach to the localisation of E/R. The retention of E/R activity in glass-mounted, frozen sections after exposure to various histochemical processes was assessed by measuring the uptake of [ $^3\text{H}$ ]  $\text{E}_2$ , well established as a valid "tracer" for E/R activity by virtue of its use in DCC and SDG assays.

Approximately 200 mg of the tissue under study was cut into cryostat sections of appropriate thickness (4  $\mu\text{m}$  or 14  $\mu\text{m}$ ). The sections were mounted on glass coverslips (22 mm x 22 mm); approximately 32 coverslips sufficed to accommodate 200 mg of tissue. After brief air-drying of the sections onto the glass, fixation, by various means, could be carried out. The treated coverslips were then divided between 2 humidifier chambers, the sections in the first chamber were flooded with a solution of [ $^3\text{H}$ ]  $\text{E}_2$  (20,000 cpm/ml) and those in the second chamber with a similar solution to which had been added an excess (25,000 pg/ml) of  $^0\text{E}_2$ . The sections were then allowed to incubate with the steroid for 2 hr. at room temperature. After incubation, the coverslips from each chamber were transferred to a small rack in which they could be immersed in various washing or "post-fixation" media.

At the end of processing, each of the 2 sets of coverslips was fragmented sufficiently to enable the whole set to be introduced into a vial of scintillator fluid. The radio-labelling of each set of frozen sections was then determined by scintillation counting. E/R activity was regarded as being present in the frozen sections if the radio-labelling of those incubated with [ $^3\text{H}$ ]  $\text{E}_2$  alone was well in excess of the radio-labelling of those co-incubated with  $^0\text{E}_2$ .

#### 4. ASSAY OF PROGESTOGEN RECEPTOR (Pg/R) ACTIVITY IN TISSUE CYTOSOLS

These assays were performed by the method of Hawkins et al. (1980), using Organon 2058 as the progestogen for receptor binding.

##### REAGENTS

These were as for E/R assay but [ $^3\text{H}$ ]  $\text{E}_2$  was replaced by tritium - labelled Organon 2058 ([ $^3\text{H}$ ] Pg) and  $^0\text{E}_2$  by organon 2058 ( $^0\text{Pg}$ ).

##### METHOD

Assay tubes for Pg/R saturation analysis were prepared, in duplicate. Each tube contained 100 pg of [ $^3\text{H}$ ] Pg and a variable mass of  $^0\text{Pg}$  (0, 50, 100, 200, 500 and 1,000 pg). The volume in each tube was made up to 1 ml with Tris/glycerol buffer. For the estimation of "non-specific binding", tubes were prepared containing 5,000 pg of  $^0\text{Pg}$ .

After mixing of the tubes, 200  $\mu\text{l}$  of the tissue cytosol under study (prepared as for E/R assay) was added to each. After 20 min., 100 pg of [ $^3\text{H}$ ] Pg was added to each of the "non-specific binding" tubes, giving sufficient time for the  $^0\text{Pg}$  to "saturate" any Pg/R in the cytosol. After further mixing, all tubes were left to incubate overnight, on ice.

The next morning, 0.5 ml of charcoal suspension was added to each tube, the contents mixed, allowed to stand for 5 min., on ice, then centrifuged at 2,500 rpm for 10 min. in the MSE, Mistral 6L centrifuge. The supernatants were transferred to plastic vials of scintillator, counted and the data analysed as for E/R assay. Pg/R concentrations were expressed in fmoles/mg of wet tissue.

5. ASSAY OF ENDOGENOUS PEROXIDASE ACTIVITY IN  
TISSUE EXTRACTS

These assays were performed by a method based on that of Lyttle and DeSombre (1977), which was modified from the original work of Himmelhoch et al. (1969). The experimental conditions and reagent concentrations employed in the assay described here were determined by a series of preliminary experiments (pages 77 - 92 ).

REAGENTS

1. 0.5M  $\text{CaCl}_2$  in Tris/HCl buffer (10mM; pH 7.2).
2. Guaiacol (O-methoxyphenol) from BDH, 40mM in the above buffer. Prepared freshly each day.
3. Hydrogen peroxide from BDH, 12.3mM in the above buffer. The concentration of the  $\text{H}_2\text{O}_2$  solution was checked daily by measurement of extinction at 240 nm ( $0.485 \pm 0.02$  with 1 cm light path).

METHOD

Tissues were harvested from the hosts and cytosol/pellet preparations produced as described with reference to E/R assay, but with the omission of monothioglycerol from the homogenisation buffer. The pellets were rehomogenised, at a concentration of 100 mg/ml (human tissues) or 300 mg/ml (rat tissues) in the 0.5M  $\text{CaCl}_2$ /Tris buffer to solubilise any peroxidase present. These homogenates were centrifuged at 25,000 rpm for 45 min. in the MSE Superspeed 50 centrifuge. The resulting supernatants were removed with a Pasteur pipette and assayed for peroxidase activity.

At room temperature, the following reagents were added to a glass cuvette of 1 cm light path: Guaiacol

(final concentration 13.3mM), hydrogen peroxide (final concentration 0.41mM) and tissue extract under study (0.02 to 0.1 ml depending on peroxidase content). The final volume was made up to 3 ml by the addition of 0.5M  $\text{CaCl}_2$ /Tris buffer. A second cuvette was prepared as a "reagent blank" by the omission of tissue extract. The reaction was commenced by the addition of the tissue extract and the "initial" rate of reaction measured by the increase in absorbance at 470 nm, over 3 minutes, between 1 and 4 minutes after starting the reaction.

Peroxidase activities were expressed in Units per g of wet tissue. One peroxidase unit was defined as the amount of enzyme required to produce an increase of one absorbance unit per min. under the assay conditions used.



## 6. HISTOCHEMICAL LOCALISATION OF ENDOGENOUS PEROXIDASE

This technique is based on that described by Graham and Karnovsky (1966). The techniques of tissue fixation described by these workers have been simplified to make the technique more suitable for light, rather than electron microscopy. (Preliminary studies indicated that fixation in ethanol rather than Karnovsky's fixative did not diminish the peroxidase-staining visible in the rat mammary tumours under study)

Blocks of rat mammary tumour measuring approximately 5 x 5 x 2 mm were mounted on metal chucks, embedded in Cryo-M-Bed (Brights), rapidly frozen using "Polar" spray and cut into 4  $\mu$ m sections on a Brights' cryostat. The sections were mounted on gelatine-coated glass slides, to improve section adhesiveness. The mounted sections were fixed for 30 min. in absolute ethanol at room temperature. After evaporation of the ethanol, the sections were flooded with DAB -  $H_2O_2$  solution (prepared by dissolving 5 mg of diaminobenzidine tetrahydrochloride from Sigma in 10 ml of 0.1 M Tris buffer, pH 7.6 plus 0.33 ml of 1%  $H_2O_2$ ). After incubation for 10 min. in this solution, the reaction was arrested by immersing the slides in tap water. The sections were then briefly counterstained in toluidine blue, dehydrated through ascending concentrations of alcohol, cleared in xylene and mounted under coverslips. Control sections were incubated with DAB solution from which  $H_2O_2$  had been omitted to identify any non-specific staining.

The stained sections were examined, by light microscopy, for intracellular, brown granules indicative of the presence of peroxidase, and were scored as follows:

- 0 : No peroxidase present.
- 1 : Peroxidase present in < 50% of cells.
- 2 : Peroxidase present in > 50% of cells.

7. SYNTHESIS OF OESTRADIOL - BSA - FLUORESCEIN AND  
BSA - FLUORESCEIN CONJUGATES

Conjugates for the histochemical localisation of E/R were prepared as described by Pertschuk et al. (1979). The conjugation procedure was based on the mixed anhydride method of Erlanger et al. (1957) and aimed to combine 4 mol. of oestradiol (linked at the C<sub>17</sub> position) and 4 mol. of fluorescein per mol. of BSA.

Oestradiol 17 $\beta$  hemisuccinate (10 mg; 30  $\mu$ moles), tri-n-butylamine (0.0075 ml; 30  $\mu$ moles) and isobutylchloro-carbonate (0.004 ml; 30  $\mu$ moles) were dissolved in 0.5 ml of anhydrous dioxane and the reaction allowed to proceed at 10°C for 45 min. BSA (135 mg) was then dissolved in 16 ml of 50% aqueous dioxane at pH 9.0 and 10°C and the above reaction mixture added, in one step, with rapid stirring. The pH was maintained at 9.0 by the addition of N. NaOH and stirring continued for 1 hr.

The reaction mixture was then transferred to dialysis tubing (Visking) and dialysed for 30 hr. against PBS, and 30 hr. against distilled water, at 4°C. The resulting solution was lyophilised to dryness. The yield of oestradiol-BSA (approx. 10 mg) was redissolved in 16 ml of 0.1 M. sodium carbonate buffer (pH 9.5) and 1 mg of fluorescein iso thiocyanate (FITC) added. The mixture was left at 0°C, for 24 hr. with gentle stirring. The resulting solution was dialysed against distilled water at 4°C for 72 hr. and again lyophilised to dryness.

The yield of oestradiol-BSA-fluorescein conjugate was dissolved in 10% ethanol in PBS at a concentration of 1 mg/ml. This stock solution was further diluted for histochemistry.

An attempt was made to confirm incorporation of oestradiol, BSA and FITC into the conjugate by scanning spectrophotometry (fig. 26 ).

A conjugate comprising BSA and FITC only was also synthesised to serve as a control for the assessment of non-specific staining of tissue sections due to binding of BSA. BSA (135 mg) was dissolved in 16 ml of sodium carbonate buffer, 13 mg of FITC was added, the mixture allowed to react and the product dialysed and lyophilised as described for the oestradiol-BSA-FITC conjugate.

## 8. SYNTHESIS OF OESTRADIOL - BSA - PEROXIDASE (HRP) CONJUGATES

Horseradish peroxidase (HRP) is a glycoprotein, of which the carbohydrate moieties are not required for enzymatic activity. Because of this structural property, the carbohydrate groups can be utilised for conjugation to various proteins including antibodies and albumin. HRP can be visualised by histochemical staining and can, therefore, serve as a tracer for such proteins in tissue sections. Two conjugates of HRP with oestradiol, via BSA, have been prepared for use in the histochemical detection of E/R as described by Walker et al. (1980).

### A. Conjugation By The Method Of Nakane and Kawaoi (1974)

In this technique, the addition of sodium periodate to HRP brings about conversion of carbohydrate groups to aldehydes which can then react with protein to form "Schiff's bases". Fluoro-dinitrobenzene (FDNB) is added to the reaction mixture in an attempt to prevent "self-coupling" and the formation of peroxidase polymers. By this method it is aimed to combine 33 moles of oestradiol (linked via the C<sub>6</sub> position) and 2 - 4 moles of HRP per mole of BSA.

HRP (type IV from Sigma), 10 mg, was dissolved in 2 ml of freshly prepared 0.3 M sodium bicarbonate solution at pH 8.1. To this solution was added 0.2 ml of a 1% solution of 1-fluoro-2,4,dinitrobenzene (FDNB) from Sigma in ethanol. The reaction was allowed to proceed for 1 hr. at room temperature, with constant stirring. To the reaction mixture was then added 2 ml of a 0.05 M solution of sodium-M-periodate (Sigma) in distilled water; mixing was continued for a further 30 min. at room temperature. During this time the mixture was seen to change to a yellow-green colour, indicative of the formation of aldehydes.

The reaction was then arrested by means of the addition of 2 ml of 0.16 M ethylene glycol in distilled water. Following this addition, mixing was continued for a further hour. The reaction mixture was then transferred to dialysis tubing (Visking) and dialysed overnight against 2 x 2 L of sodium carbonate buffer (0.01 M; pH 9.5) at 4°C.

The reaction mixture (comprising aldehydes of HRP) was then removed from the dialysis tubing and 5 mg of 17 $\beta$  oestradiol-6-O-carboxymethyloxime-BSA (from Steraloids, containing 33 moles of steroid per mole of BSA) added, by sprinkling with rapid mixing. After dissolution of the protein-steroid conjugate, gentle mixing was continued for 3 hours at room temperature. This reaction resulted in formation of a "Schiff's base" which was stabilised by the addition of 5 mg of sodium borohydride (Sigma). Following this addition, the mixture was allowed to stand for 3 hours at 4°C.

The reaction product was then dialysed against 3 x 2 L of PBS at 4°C over a period of 72 hours. The product was then applied, in 1 ml aliquots, to an 85 x 1.5 cm column of Sephadex G100 equilibrated with PBS. Fractions (1.5 ml) were collected and the absorbance of each fraction at 280 nm measured using an SP200 ultraviolet spectrophotometer. The fractions forming the first peak (see fig. 3) were pooled, and were taken to comprise the yield of oestradiol-BSA-HRP conjugate. The remaining fractions, containing residual, unconjugated BSA-oestradiol and HRP were discarded. The yield was frozen in 1 ml aliquots and stored at -20°C for subsequent use.

#### B. Conjugation By The Method Of Avrameas and Ternynck (1971)

In this method, glutaraldehyde rather than periodate is used to form aldehyde groups with HRP for reaction with proteins. The spacial configuration of the glutaraldehyde

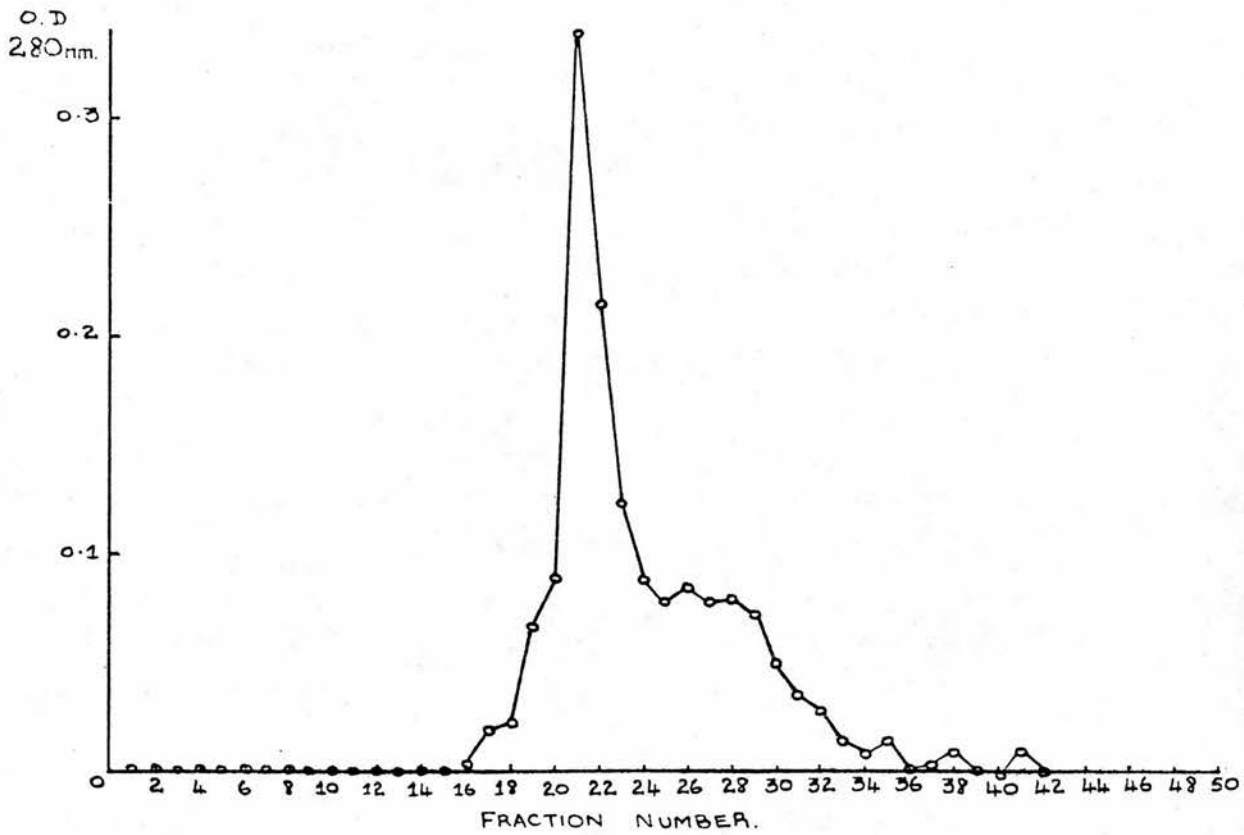


Figure 3.

Elution pattern of 6-keto-oestradiol-BSA conjugated with horse-radish peroxidase. Chromatography was carried out on a 85x1.5 cm. column of Sephadex, flow rate approx. 10ml./hr. Fractions 20 to 25 were pooled as representing the yield of oestradiol-BSA-peroxidase conjugate.

derivative of HRP is, reportedly, such that "self-coupling" and thus polymer formation is minimised. By this method, it is aimed to combine 33 moles of oestradiol and 1 mole of HRP per mole of BSA.

HRP (type IV from Sigma), 10 mg, was dissolved in 0.2 ml of 0.1 M phosphate buffer, pH 6.8, containing 1.25% glutaraldehyde. The solution was allowed to stand for 18 hr. in a cool room and was then filtered through a 60 x 1.2 cm column of Sephadex G-25 equilibrated with 0.15 M NaCl. The brown fractions, containing the "activated" peroxidase were pooled and were concentrated to 1 ml by transferring to a length of dialysis tubing and immersing in Sephadex powder.

To the concentrated solution was added 1 ml of 0.15 M NaCl containing 4 mg of 17 $\beta$ -oestradiol-6-O-carboxymethyloxime-BSA (Steraloids), followed by the addition of 0.1 ml of 1 M carbonate-bicarbonate buffer, pH 9.5. After 24 hours at 4°C, 0.1 ml of 0.2 M solution of lysine was added and the mixture allowed to stand for an additional 2 hr.

The preparation was then extensively dialysed against several changes of PBS at 4°C and was again concentrated to a small volume using Sephadex. The product was filtered through a 100 x 1.2 cm column of Sephadex G-200 and the absorbances of the resulting fractions at 280 nm (protein maximum) and at 430 nm (peroxidase maximum) determined. The fractions comprising the main peak (Figure 3a) were pooled and were again concentrated to a volume of approximately 4 ml for histochemical use.



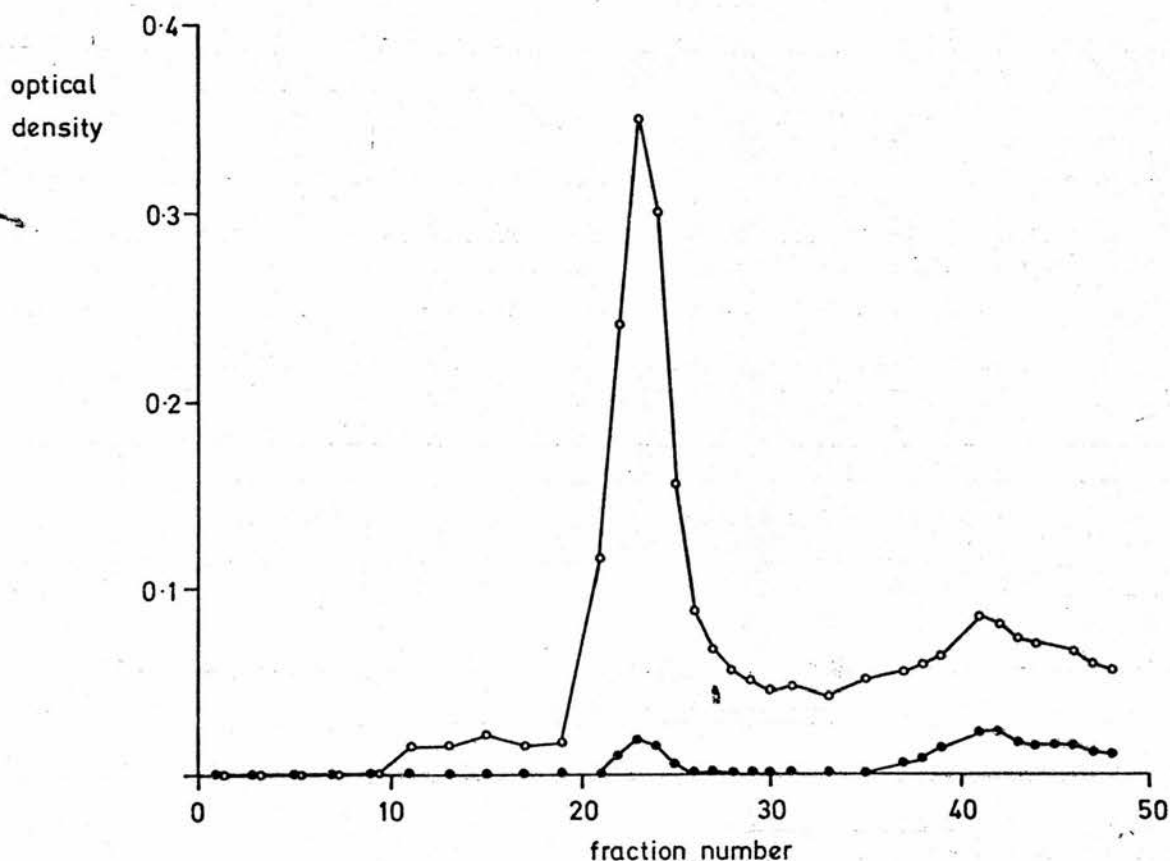


Figure 3a.

Elution pattern of 6-Keto-oestradiol-BSA conjugated with horseradish peroxidase by the method of Avrameas & Ternynck. Chromatography was carried out on a 100x1.2cm. column of Sephadex G200.

The upper line indicates absorbance at 280 nm. (protein maximum) and the lower line represents absorbance at 430nm. (peroxidase maximum). Fractions comprising the main peak were pooled as representing the yield of conjugate.

9. HISTOCHEMICAL METHOD FOR THE DETECTION OF E/R  
USING FLUORESCCEIN TRACER

This technique was described by Pertschuk et al. (1979) and has been used by the author in an attempt to reproduce the results described by these workers.

Frozen sections (4  $\mu$ m) of the tissue under study were cut using a Bright's cryostat. The sections were mounted on gelatin-coated slides and allowed to air-dry briefly to ensure adhesion of the sections to the slides. Serial sections were then incubated in a humidifier chamber, at room temperature, for 2 hr. in each of the following solutions:

1. Oestradiol-BSA-fluorescein (50  $\mu$ g/ml) in PBS containing 10% ethanol.
2. The "control" conjugate, BSA-fluorescein, also at a concentration of 50  $\mu$ g/ml.
3. Oestradiol-BSA-fluorescein (50  $\mu$ g/ml) plus an excess of a competitive "receptor-blocker", CI628 (100  $\mu$ g/ml).

Approximately 3 drops of the appropriate solution was needed to cover each section.

After incubation, excess conjugate solution was rinsed off with PBS from a wash-bottle. The sections were then fixed by immersing for 10 min. in a mixture of equal volumes of ethanol and acetone. Following fixation the sections were washed for a total of 30 min. by immersing in 3 changes of PBS. After a final rinse in distilled water the sections were mounted in buffered glycerol (7 - 8 parts PBS, 2 - 3 parts glycerol), under coverslips for fluorescence microscopy.

The sections were viewed using a Zeiss large research microscope under incident UV light using tube head filter no. 53.

10. HISTOCHEMICAL METHOD FOR THE DETECTION OF E/R USING  
PEROXIDASE TRACER

This technique was described by Walker et al. (1980) and has been used by the author in an attempt to reproduce the results described by these workers.

Frozen sections (4  $\mu$ m) of the tissue under study were cut using a Bright's cryostat. The sections were mounted on plain glass slides and allowed to air-dry briefly to ensure adhesion of the sections to the slides. The sections were then fixed by immersing in acetone, at room temperature, for 4 min. Serial sections were then incubated in a humidifier chamber, at room temperature, for 2 hr. in each of the following solutions:

1. 17- $\beta$ -oestradiol-6-O-carboxymethyloxime-BSA-horse radish peroxidase in PBS at a dilution of between 1/8 and 1/20 of the concentration obtained at synthesis. (The required dilution was determined empirically by examination of preliminary sections).
2. A similar solution of conjugate plus an excess of a competitive "receptor blocker", tamoxifen or diethylstilboestrol in saturated solution.

After incubation, excess conjugate solution was rinsed off with PBS from a wash bottle and the slides then extensively washed in 3 changes of PBS for a total of 30 min. In order to visualise any peroxidase-labelling of the sections, they were incubated with a solution of diaminobenzidine plus hydrogen peroxide (see method 6), washed and counter-stained with haematoxylin. The sections were then dehydrated, cleared and mounted as described in method 6 and viewed under the light microscope.

PART III

RESULTS OF STUDIES CONCERNING  
PEROXIDASE AS A MARKER FOR HORMONE-DEPENDENCE  
&  
THE HISTOCHEMICAL LOCALISATION OF OESTROGEN RECEPTORS

# I. PRELIMINARY EXPERIMENTS TO ESTABLISH A BIOCHEMICAL ASSAY FOR PEROXIDASE ACTIVITY

## A. Determination Of Optimal Substrate Concentrations

The kinetics of enzyme-catalysed reactions are such that, at low substrate concentrations, the initial rate of reaction,  $v$ , is directly related to substrate concentration,  $[s]$ . At a certain, higher substrate concentration,  $[S]$ , all the available enzyme is "saturated" with substrate and the maximum initial velocity,  $V$ , for those reaction conditions (i.e. temperature, pH, enzyme concentration) is reached. When the initial rate of such a reaction is being used as a measure of enzyme activity it is desirable that substrate concentrations should exceed  $[S]$  since in these circumstances the rate of reaction is uninfluenced by minor changes in substrate concentration and is a true reflection of the amount of enzyme present. Preliminary experiments were performed to determine appropriate substrate concentrations, according to this principle, for the assay of peroxidase.

The assay conditions were as described in Method 5, but the cuvette mixture comprised a fixed concentration of HRP (equivalent to 20  $\mu$ l of a 0.05 mg/ml solution in a final volume of 3 ml) and variable concentrations of substrates (guaiacol and  $H_2O_2$ ). For determination of the optimal concentration of guaiacol, the  $H_2O_2$  concentration was maintained at 0.41 mM and six concentrations of guaiacol (equivalent to 0.1 - 2 ml of a 40 mM solution in a final volume of 3 ml) were tested. For determination of the optimal concentration of  $H_2O_2$ , the guaiacol concentration was maintained at 13.3 mM and six concentrations of  $H_2O_2$  (equivalent to 0.01 - 0.2 ml of a 12.3 mM solution in a final volume of 3 ml) were tested.

## RESULTS

### a) Optimal Concentration Of Guaiacol

The initial rate of reaction for each concentration of guaiacol tested is shown in Figure 4. The initial rate increased with increasing concentration of guaiacol to a maximum at a concentration equivalent to 0.5 ml of the stock solution in a final volume of 3 ml. Thereafter, further increases in concentration of guaiacol failed to cause any increase in the rate of reaction, indicating saturation of the enzyme with substrate. A volume of 1.0 ml of 40 mM guaiacol (equivalent to a working concentration of 13.3 mM), which lay well onto the "plateau" region of the curve, was selected for the routine assay.

The same data plotted by the method of Lineweaver and Burke (1934), to yield a straight line, are shown in Figure 5. A value for the Michaelis constant ( $K_m$ ) for horseradish peroxidase with guaiacol as substrate was derived from this graph. The value of  $K_m$  was equivalent to a concentration of 1.5 mM guaiacol.

### b) Optimal Concentration Of $H_2O_2$

The initial rate of reaction for each concentration of  $H_2O_2$  tested is shown in Figure 6. The presence of a "plateau" region on the curve, within the range of concentrations of  $H_2O_2$  tested, was not as evident as on the curve for guaiacol. A quantity of 0.1 ml of 12.3 mM  $H_2O_2$  (equivalent to a working concentration of 0.41 mM) was selected, somewhat empirically, for the routine assay.

The same data plotted according to Lineweaver and Burke (1934) are presented in Figure 7. From this graph, a value for  $K_m$  with respect to  $H_2O_2$  of 0.41 mM was derived.

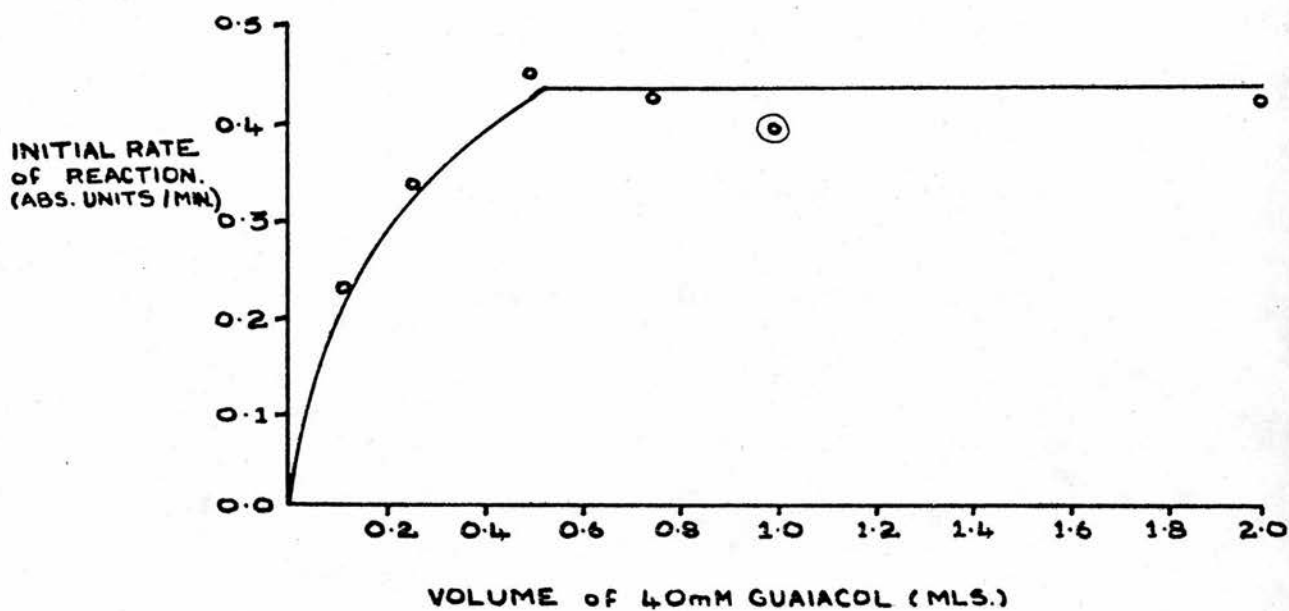


Figure 4.

Initial rate of peroxidase-catalysed reaction with varying concentrations of substrate (guaiacol).

Each symbol represents the value obtained in a single assay.

The double circle indicates the concentration chosen for the routine assay.



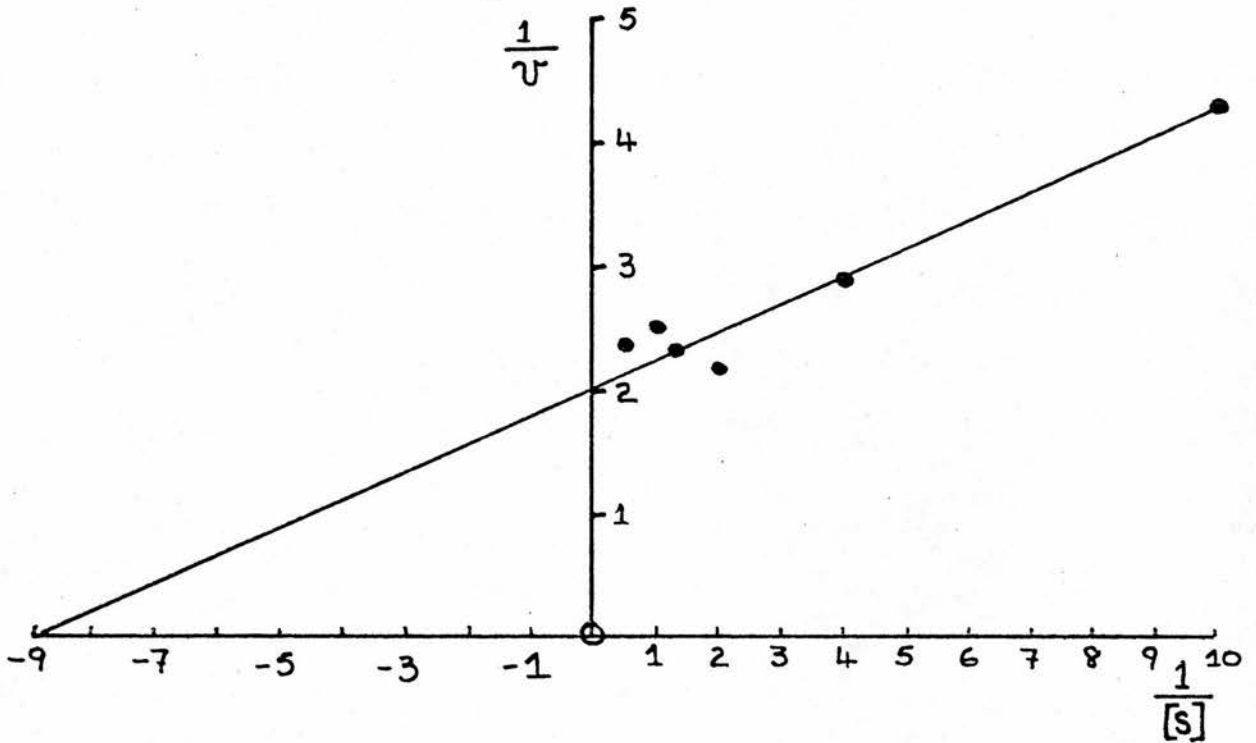


Figure 5.

Lineweaver-Burke plot of data in fig.4.

$v$  = Initial rate of reaction ( absorbance units / min.)

$(s)$  = concentration of guaiacol expressed as mls. of a 40mM. solution in a final cuvette volume of 3 mls.

$K_m$  calculated from this graph = 1.5mM. guaiacol.

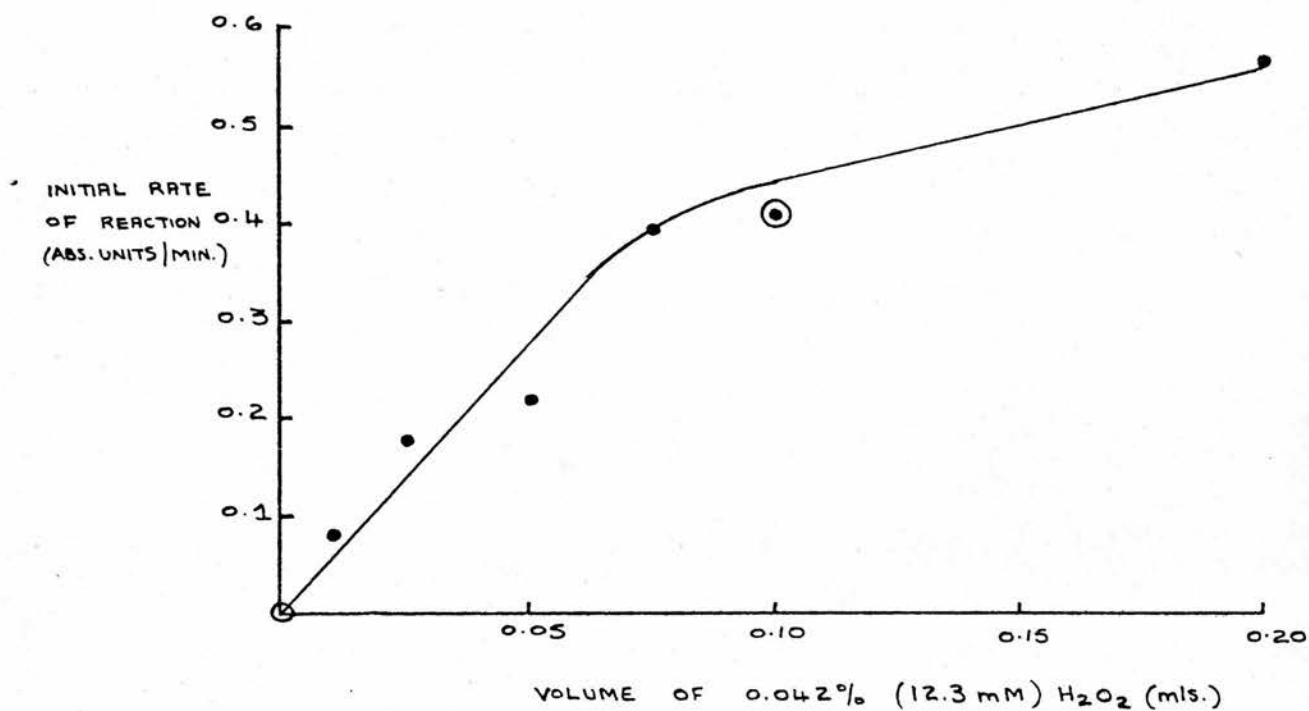


Figure 6.

Initial rate of peroxidase-catalysed reaction with varying concentrations of substrate ( $\text{H}_2\text{O}_2$ ).

Each symbol represents the value obtained in a single assay.

The double circle indicates the concentration chosen for the routine assay.

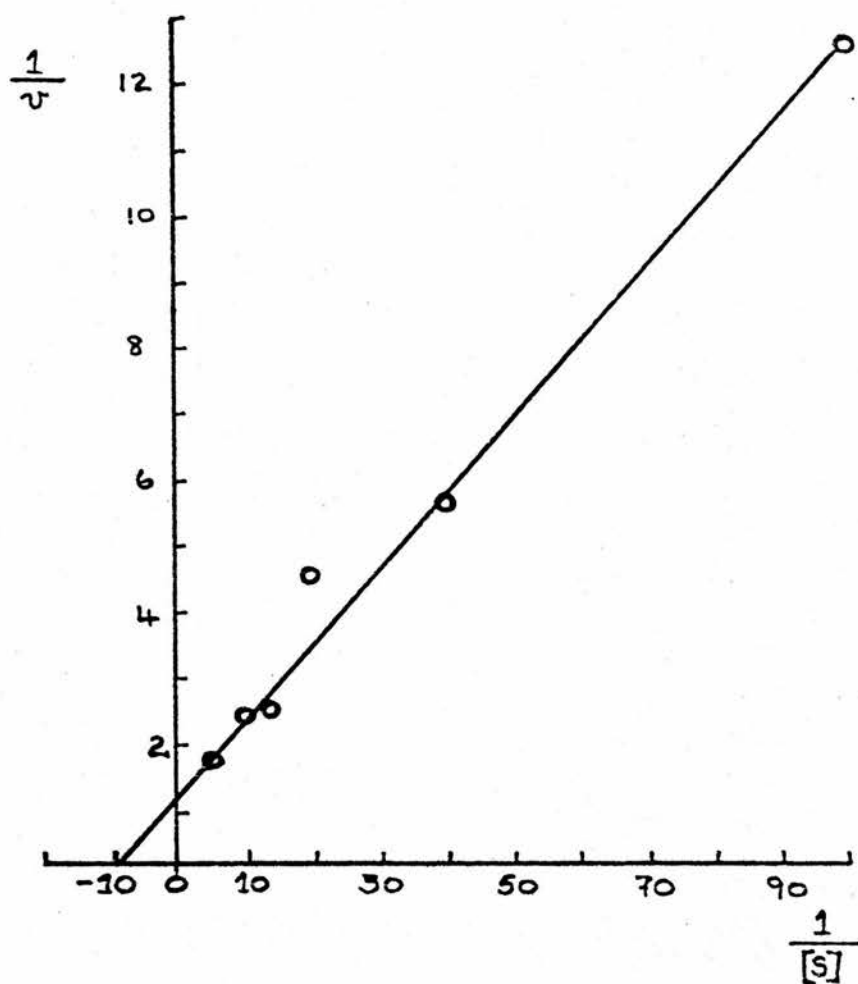


Figure 7.

Lineweaver-Burke plot of data in fig. 6.

$v$  = Initial rate of reaction in absorbance units / min.

$(s)$  = Concentration of  $H_2O_2$  expressed as mls. of a 12.3 mM. solution in a final cuvette volume of 3 mls.

$K_m$  calculated from this graph = 0.41 mM.  $H_2O_2$ .

B. Determination Of Optimal Method Of Measuring  
Initial Rate Of Reaction

This assay system is based upon the principle that enzyme activity is directly proportional to the "initial rate of reaction" under constant reaction conditions. The rate of reaction is reflected by the rate of appearance of the coloured reaction product (guaiacol dehydrogenation product) which is, in turn, reflected by the rate of increase in absorbance at 470 nm. It is impossible, in practice, to measure what is truly the initial rate of reaction, as mixing of the cuvette contents and manipulation of the spectrophotometer take a finite period of time. An attempt has been made to determine the method of assessing "initial" rate of reaction which, in practice, gives the best correlation with peroxidase activity.

Using the assay conditions and reagent concentrations as described in Method 5, six different concentrations of horseradish peroxidase (0.02 ml to 0.12 ml of a 0.005 mg/ml solution) were assayed. For each of the six concentrations the absorbance at 470 nm was measured at one minute intervals from 0 to 5 minutes after starting the reaction. Using these data 3 different assessments of "initial" rate of reaction were calculated for each HRP concentration:

1. Increase in absorbance from 0 to 1 minute.
2. Mean increase per minute from 0 to 3 minutes.
3. Mean increase per minute from 1 to 4 minutes.

RESULTS

The time course of change in absorbance is shown for each of the six concentrations of HRP studied in Figure 8. These curves show that for all the concentrations of HRP, the rate of reaction was linear between 0 and 5 minutes.

In Figure 9, the 3 different assessments of "initial" rate of reaction for each of the 6 HRP concentrations are presented. Study of the graph reveals that Method 3 (i.e. mean increase in absorbance per minute from 1 to 4 minutes after starting the reaction) best approached a linear relationship between HRP concentration and initial rate of reaction. This method was, therefore, selected for the routine assay system.

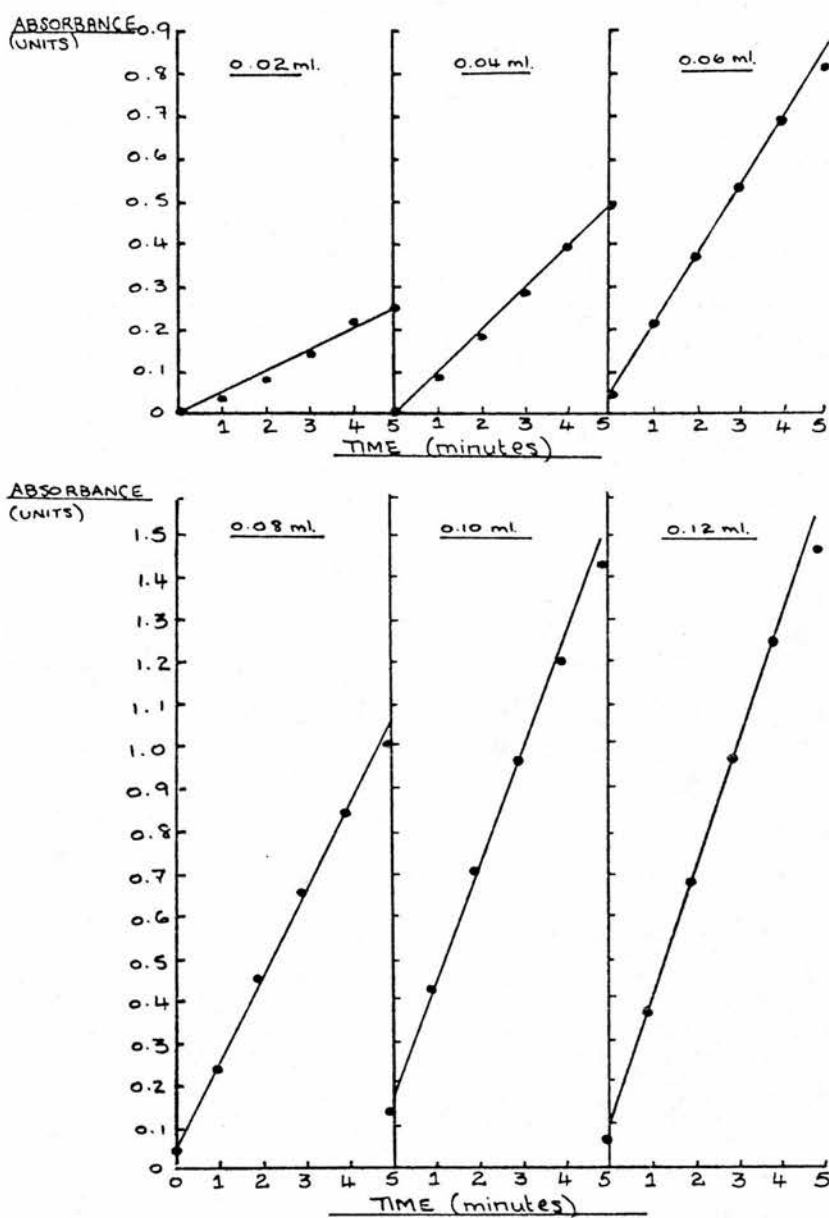


Figure 8.

Absorbance / Time graphs for each of 6 concentrations of HRP. Concentration is expressed in terms of mls. of a 0.005 mg/ml. solution of HRP. in a final cuvette volume of 3 mls.

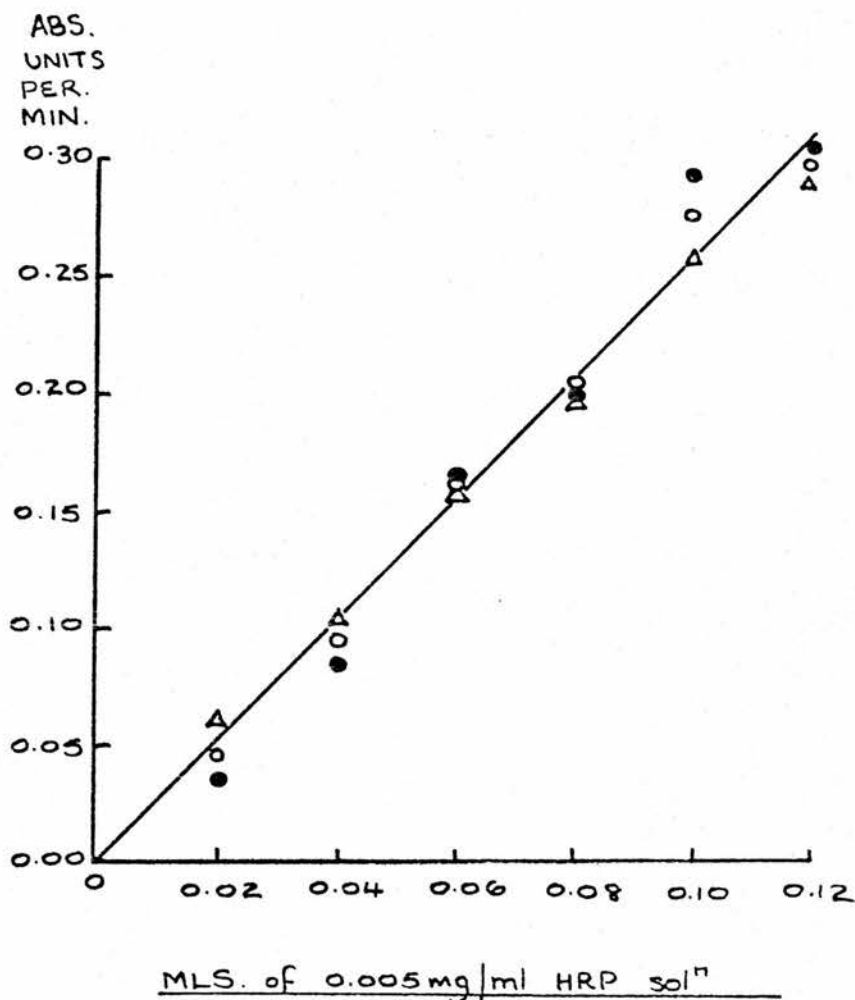


Figure 9.

Initial rate of reaction ( absorbance units/min. )  
calculated in 3 different ways for each of 6 concentrations  
of HRP.

Each symbol indicates the result of a single assay.

- = Increase in absorbance/min. calculated from 0-1 min.
- = " " " " " 0-3 min.
- Δ = " " " " " 1-4 min.



C. Validation Of Assay Using Known Concentrations Of  
HRP And Uterine Peroxidase

Further experiments were performed to confirm the existence of a linear relationship between "initial rate of reaction" and enzyme concentration for both horseradish and uterine peroxidases over the range of concentrations likely to be encountered in subsequent studies. Seven concentrations of HRP (from 0.01 - 0.04 ml of a 0.05 mg/ml solution in a final volume of 3 ml) were assayed under the standard conditions. Similarly, eight concentrations of an extract prepared from rat uterus were examined.

RESULTS

The relationships between "initial rate of reaction" and enzyme concentration for HRP and for uterine peroxidase are presented in Figures 10 and 11. In the case of HRP, a linear relationship held throughout the range of concentrations tested. For the uterine extract, the relationship was linear up to a working concentration of approximately 3% extract, equivalent to a volume of 0.09 ml of extract in a cuvette volume of 3 ml.

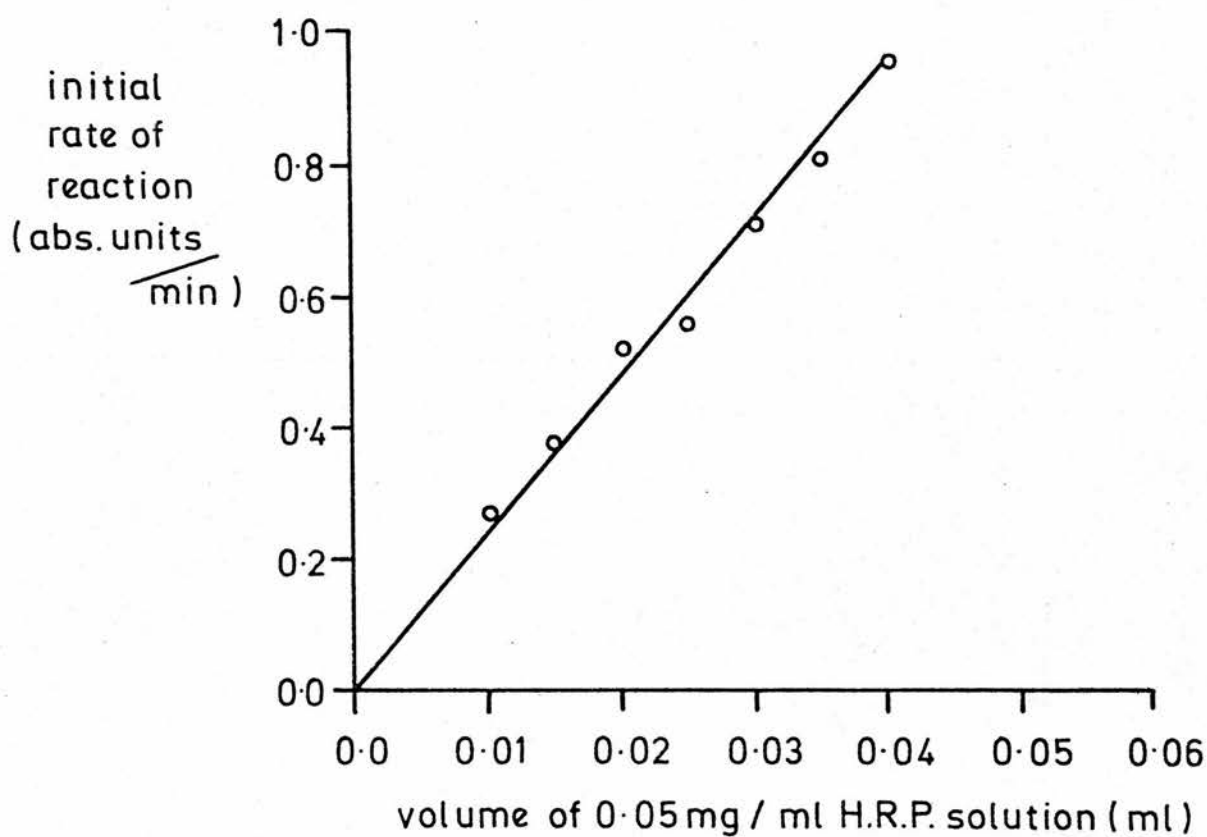


Figure 10.

The relationship between initial rate of reaction, as measured under routine assay conditions, and known concentrations of HRP.

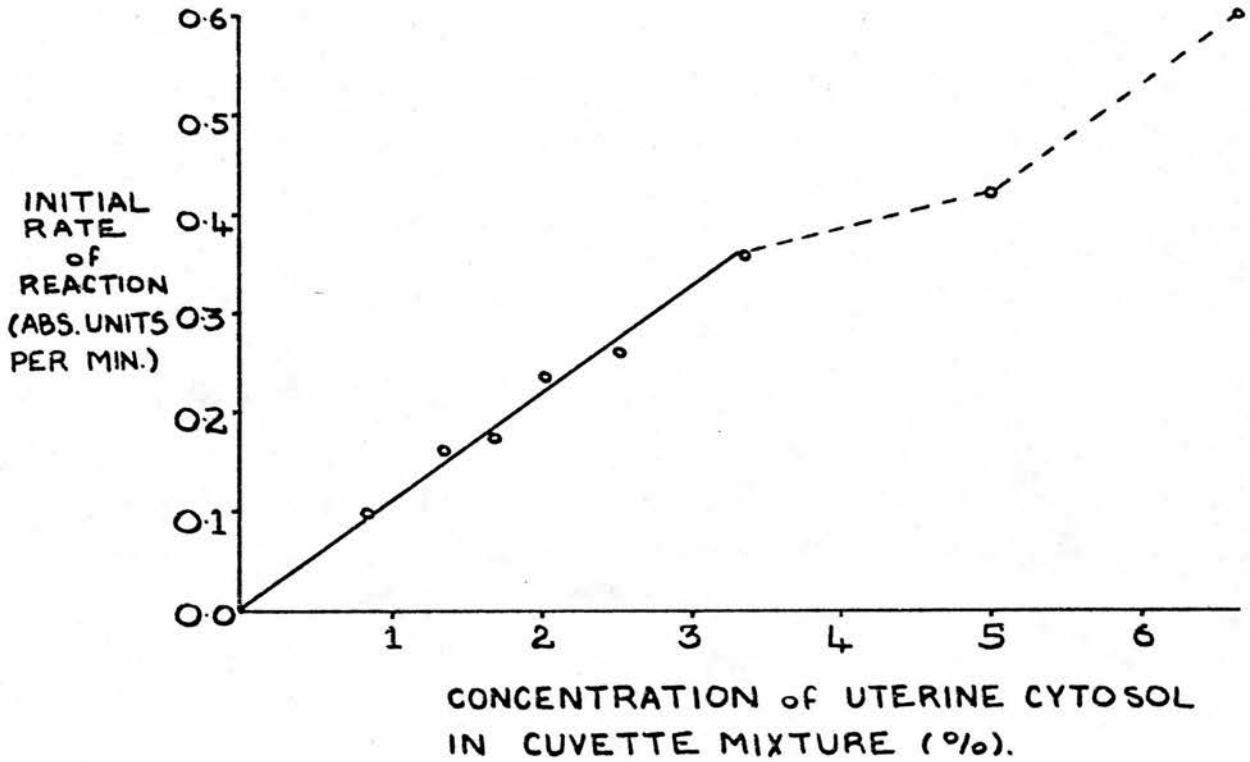


Figure 11.

The relationship between initial rate of reaction, as measured under routine assay conditions, and known concentrations of rat uterine extract.

D. Determination Of The Precision Of The Assay

A study was undertaken to determine the intra-assay precision of the peroxidase assay under the chosen conditions (Method 5). Six aliquots (0.06 ml) of a 0.005 mg/ml solution of HRP were assayed, in succession, on the same day and using the same reagent preparations, by the routine method.

RESULTS

The "initial rates of reaction" were as follows: 0.148, 0.142, 0.147, 0.163, 0.158, and 0.169. The assay precision was calculated from these data using the formula:

$$\text{Precision} = \frac{\text{standard deviation}}{\text{mean}} \times 100\%$$

The intra-assay precision was found to be 6.7%.

E. Determination Of The Storage Properties Of Tissue  
Peroxidase

Since it was not always possible to assay tissues immediately after removal from the hosts, such tissues were stored in liquid nitrogen until a convenient time for assay. A preliminary study was undertaken to investigate the stability of tissue peroxidase (from rat uterus) upon storage in liquid nitrogen. Peroxidase was extracted from a rat uterus as described under Method 5. Aliquots of this extract were frozen in liquid nitrogen and removed for assay after various periods of storage: 0, 2, 7, 16 and 33 days.

RESULTS

The peroxidase activity of the uterine extract, expressed in units/g of parent tissue, after a variable storage period is shown in Figure 12. In the fresh extract, peroxidase activity was 95 U/g. Activity apparently fell to 85 U/g after 2 days of storage but longer periods of storage resulted in no further diminution of activity, suggesting that tissues can be stored, in this way, with little or no loss of peroxidase activity.

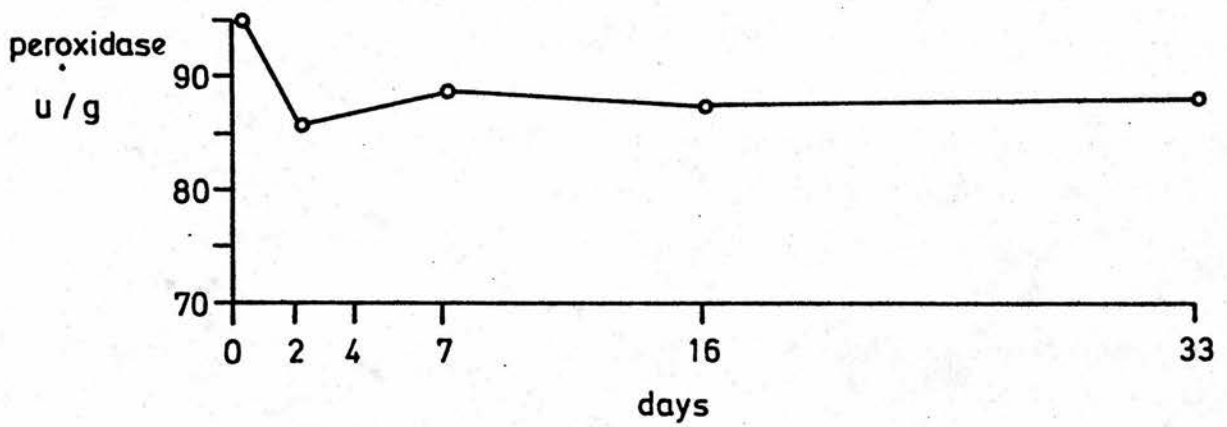


Figure 12.

Peroxidase activity detectable in aliquots of a rat uterine extract after various periods of storage in liquid nitrogen.

## II. RELATIONSHIPS BETWEEN LEVELS OF PEROXIDASE, E/R AND Pg/R IN HEALTHY RAT TISSUES

A study was made of the three postulated markers for hormone-dependence (peroxidase, E/R and Pg/R) in a range of healthy tissues of the rat. The following tissues were selected for study to provide a range of classical oestrogen-target and non-target tissues: uterus and mammary gland excised from animals in various reproductive states, liver, kidney, prostate, testis, lung, spleen and erythrocytes. The tissues were harvested from the host animals, extracted and assayed for peroxidase, E/R and Pg/R as described under the appropriate "Methods".

### RESULTS

The levels of peroxidase detected in the 15 tissues studied are presented in ascending order in Figure 13. The levels of E/R and Pg/R in the corresponding tissues are also included for comparison. Peroxidase was detectable in all tissues examined with the exception of liver and kidney. In peroxidase-positive tissues, the range of activities was very wide, from 0.1 u/gm in lactating mammary gland to 95 u/gm in uterus during oestrus.

E/R was found to be even more widely distributed than peroxidase, being absent only from erythrocytes. In those tissues in which E/R was present, levels ranged from 0.2 fmoles/mg in mammary gland during oestrus to 33 fmoles/mg in the uterus of a lactating animal.

Pg/R was much less widely distributed, being detectable only in uterus, lung and kidney. Levels were relatively low (0.72 and 1.32 fmoles/mg) in lung and kidney, but ranged from 13 to 98 fmoles/mg in uterine specimens. In parallel with peroxidase activity, Pg/R content was greatest in uterus during oestrus, and relatively low in the uterus from a lactating animal.



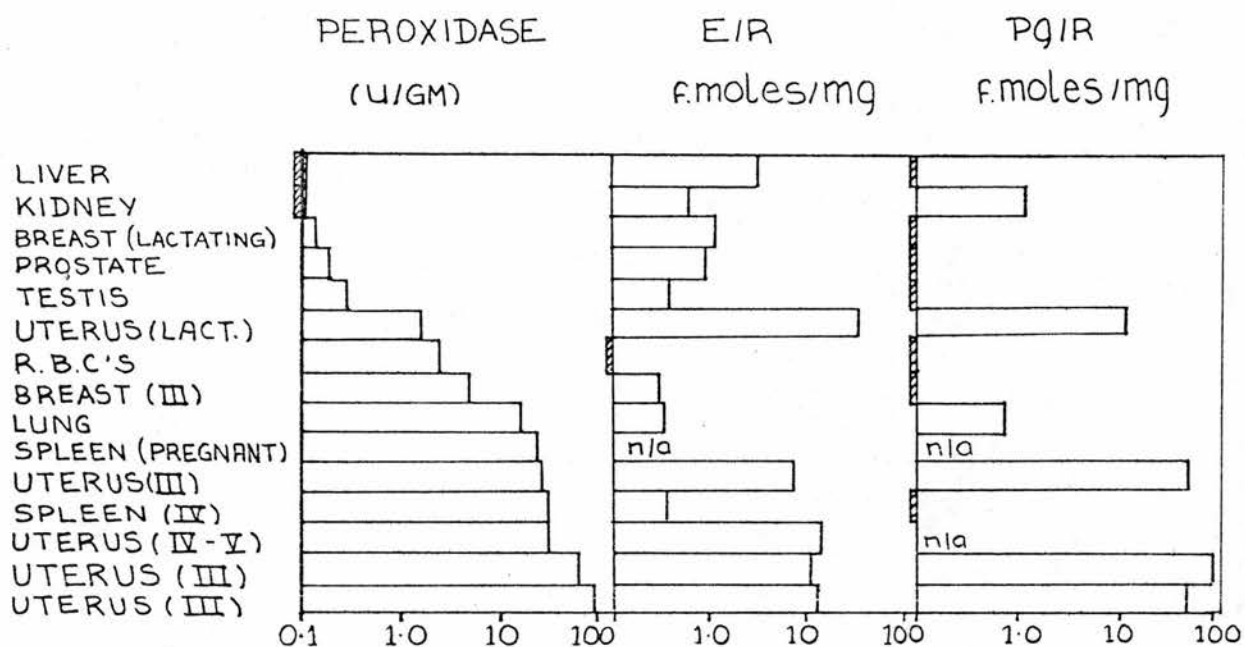


Figure 13.

Concentrations of peroxidase, E/R and Pg/R in a range of healthy rat tissues.

Each block represents the mean of duplicate estimations on a single tissue extract.

Hatched blocks indicate that the particular protein was undetectable.

n/a indicates that no result is available.

Roman numerals indicate the stage in the oestrus cycle of the rat under study.

I=proestrus; II=oestrus; III=metoestrus; IV=dioestrus

V= intermediate between IV & I.

### III. COMPARISON OF PEROXIDASE, E/R AND Pg/R AS MARKERS FOR HORMONE - DEPENDENCE IN RAT MAMMARY TUMOUR MODELS

A study was made of levels of the three postulated markers for oestrogen-dependence (E/R, Pg/R and peroxidase) in rat mammary tumours which serve as models for hormone-dependent and -independent growth. In total, 44 tumours were examined; of these, 24 were DMBA-induced tumours (models for hormone-dependent growth) and 20 were transplantable tumours of TG3 and TG5 lines (models for hormone-independent growth). The tumours were harvested from the host animals, extracted and assayed for peroxidase, E/R and Pg/R as described under the appropriate "Methods". The Wilcoxon Rank Sum Test was used to analyse the differences between levels in hormone-dependent and -independent tumours for each "marker" protein.

### RESULTS

The peroxidase content of each of the 24 "hormone-dependent" and each of the 20 "hormone-independent" tumours is shown in Figure 14. Peroxidase was undetectable in 6 of the "hormone-independent" group but in only 2 of the "hormone-dependent" group. The median level in the former group was only 0.48 u/g wet weight, but was 2.8 u/g wet weight in the latter. Overall, there was a significant difference between the levels in the two groups ( $p < 0.001$ , Wilcoxon Rank Sum Test). Nevertheless, there was a considerable overlap between the ranges of peroxidase activities in the hormone-dependent and -independent groups.

Figures 15 and 16 show the levels of E/R and Pg/R in a sample of 12 tumours from each of the two groups. For E/R, the median value in the hormone-dependent tumours

was 4.5 fmoles/mg wet weight, and in the hormone-independent group, only 0.3 fmoles/mg wet weight. There was no overlap whatsoever between the ranges of E/R levels in the two groups.

In the case of Pg/R, the median value in the hormone-dependent group was 24 fmoles/mg wet weight, and in the hormone-independent group, less than 0.1 fmoles/mg wet weight. Again, there was no overlap between the ranges of levels in the two groups, with the exception of a single DMBA-induced tumour in which Pg/R was undetectable.

It should be noted that although the hormone-independent, transplantable tumours have been considered as one group, within this group, the TG-3 line consistently yielded higher levels of each marker than did the TG-5 group.

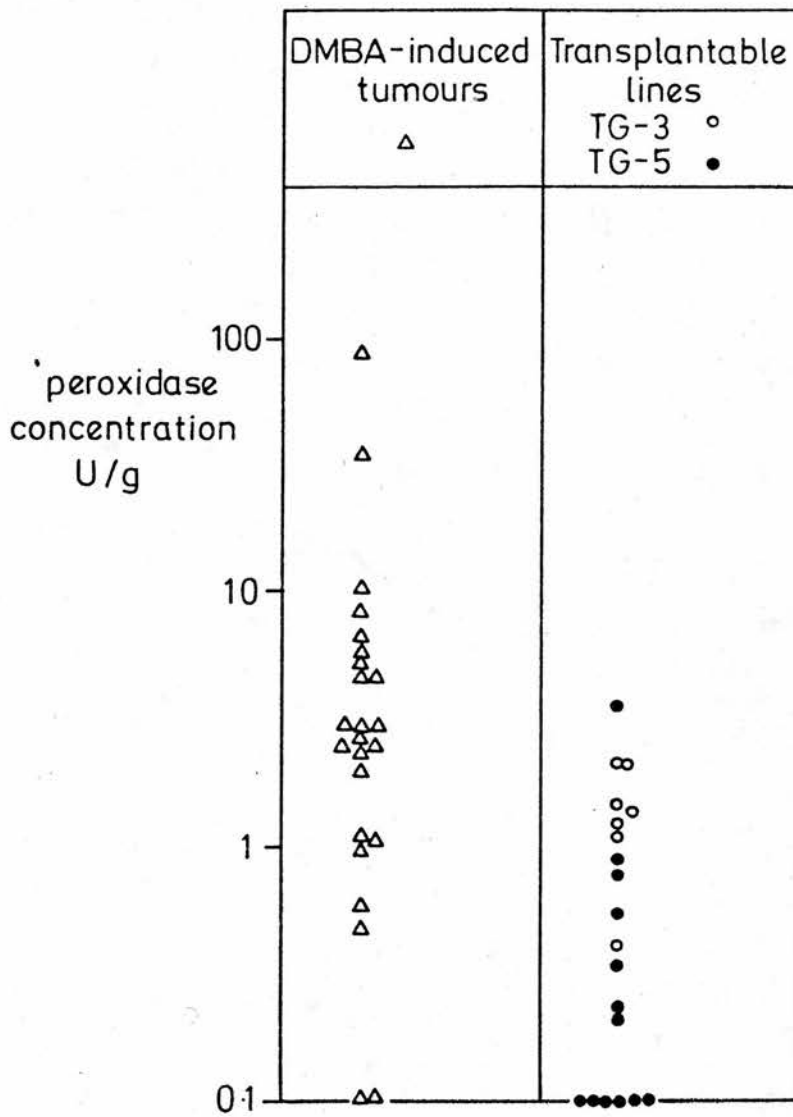


Figure 14.

Endogenous peroxidase content in rat mammary tumours which serve as models for hormone-dependent and -independent growth. Each symbol represents one tumour.

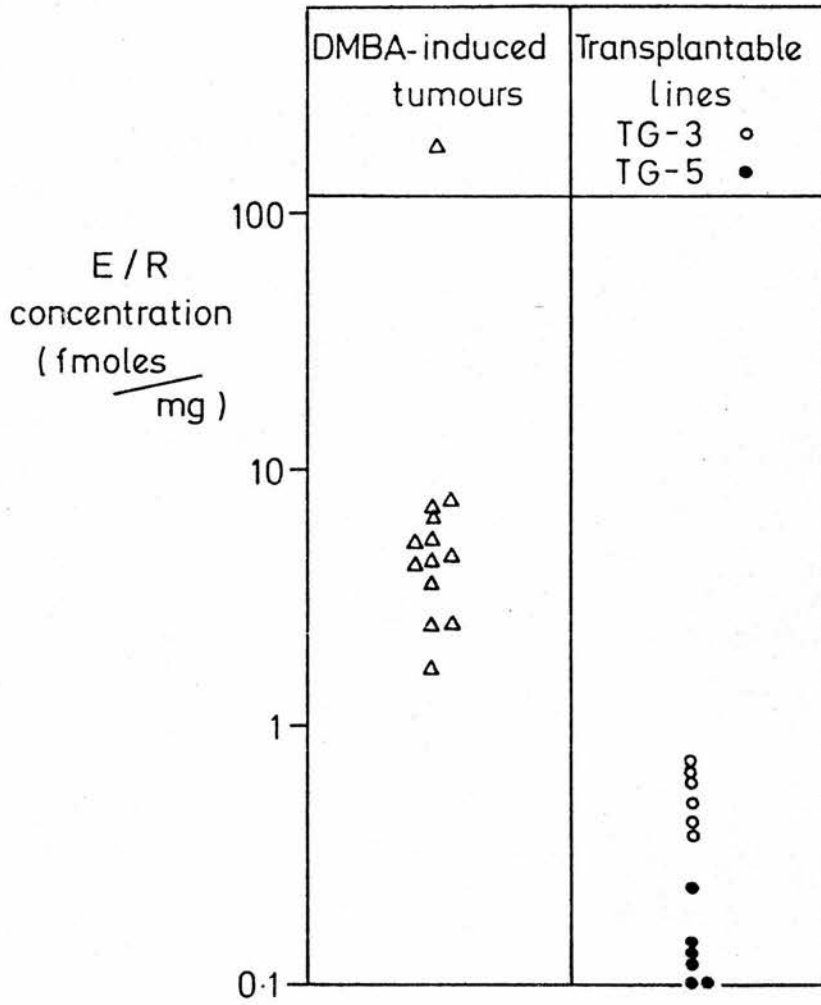


Figure 15.

E/R content in rat mammary tumours which serve as models for hormone-dependent and -independent growth.

Each symbol represents one tumour.



#### IV. THE RELATIONSHIP BETWEEN PEROXIDASE ACTIVITY AND E/R STATUS IN HUMAN MAMMARY TUMOURS

A pilot study was undertaken in an attempt to identify any relationship between levels of the postulated marker for hormone-dependence, endogenous peroxidase, and the established marker, E/R, in human mammary tumours. In total, 17 tumours were studied. These had been obtained at operation up to 2 years previously, and had been stored in liquid nitrogen until the time of assay. Tumour extracts were prepared and assayed for peroxidase and for E/R as outlined in the "Methods" section. Information regarding response to endocrine therapies was available in 3 of the 17 cases.

#### RESULTS

Of the 17 tumours examined, 6 (35%) contained less than 0.25 fmoles/mg wet weight of E/R and were designated E/R-"poor". Peroxidase was undetectable in 4 of the 11 E/R-"rich" tumours, and in 2 of the 6 E/R-"poor" tumours. The levels of E/R and peroxidase in each of the 17 tumours are presented in graphical form in Figure 17. There is, clearly, no correlation between the presence or absence of the two proteins, nor between their absolute levels.

Of the three patients who have subsequently received endocrine therapies, 2 responded and one failed to respond. The tumours of the 2 who responded to therapy were positive for both E/R and peroxidase. The tumour of the patient who failed to respond was E/R-poor, but contained one of the highest levels of peroxidase (6.8 u/g wet weight).



# E/R CONCENTRATION AND PEROXIDASE ACTIVITY IN 17 HUMAN MAMMARY CARCINOMAS

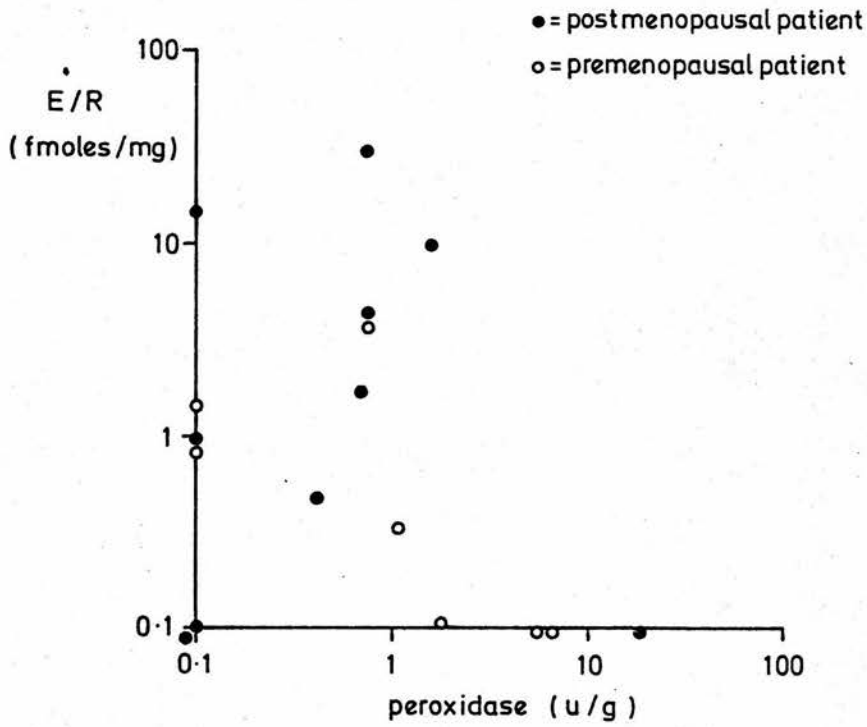


Figure 17.

The relationship between peroxidase activity and E/R content in 17 human breast cancers.

V. A COMPARISON BETWEEN THE PEROXIDASE ACTIVITY OF  
RAT MAMMARY TUMOURS AS ASSESSED BIOCHEMICALLY  
AND BY A SIMPLE HISTOCHEMICAL TECHNIQUE

Most published studies concerning peroxidase as a marker for oestrogen-dependence have employed a biochemical assay system similar to the guaiacol method described in the present work. Histochemical techniques for the detection of peroxidase are also available, and it has been suggested that such techniques might provide the basis for a simple determinant of hormone-dependence, visible at the light microscope level (DeSombre et al., 1975). In the present study, the peroxidase activity of rat mammary tumours of varying hormone-dependency was assessed by means of a very simple histochemical staining technique. It was felt that if the results obtained with this technique correlated with those of the more complex guaiacol assay, then it might prove to be of practical use as a test of hormone-dependence.

In total, 42 rat mammary tumours were studied. Of these, 26 were DMBA-induced tumours (largely hormone-dependent) and 16 were transplantable tumours of TG3 or TG5 lines (hormone-independent). The tumours were harvested from the host animals, a portion of each was frozen and processed histochemically (Method 6) and a further portion was homogenised and assayed biochemically (Method 5) for peroxidase activity.

### RESULTS

The peroxidase content of each hormone-dependent tumour and of each hormone-independent tumour, as assessed by the biochemical assay, is shown in Figure 18. The median value for the hormone-dependent tumours was 3.75 U/g, and for the hormone-independent tumours was only 0.26 U/g. The difference between the two groups was significant (Wilcoxon Rank Sum Test,  $p = 0.01$ ).

The score allocated to each hormone-dependent tumour and to each hormone-independent tumour on the basis of histochemical staining for peroxidase is shown in Table 6. No peroxidase staining was visible in 12 of 26 hormone-dependent tumours, but staining was visible in all but one of the hormone-independent group. This difference is significant ( $\chi^2 = 5.63$ ;  $p < 0.05$ ).

Overall, 13 of the 42 tumours exhibited no peroxidase staining, and the remaining 29 were judged histologically positive for peroxidase. The results of biochemical assay for peroxidase for each of the histologically negative and for each of the histologically positive tumours are shown in Figure 19. Paradoxically, the median biochemical assay result for the histologically negative tumours was 5 U/g, whereas that for the histologically positive tumours was only 0.55 U/g. This difference is significant (Wilcoxon Rank Sum Test  $p = 0.01$ ).



Table 6.

Histochemical scores of peroxidase activity in 26 hormone-dependent and 16 hormone-independent rat mammary tumours.

SCORE	HORMONE-DEPENDENT	HORMONE-INDEPENDENT
0	12	1
1	11	14
2	3	1
TOTAL	26	16

$\chi^2=5.63$ ;  $p<0.05$  for the difference between the number with absent peroxidase in the two groups.

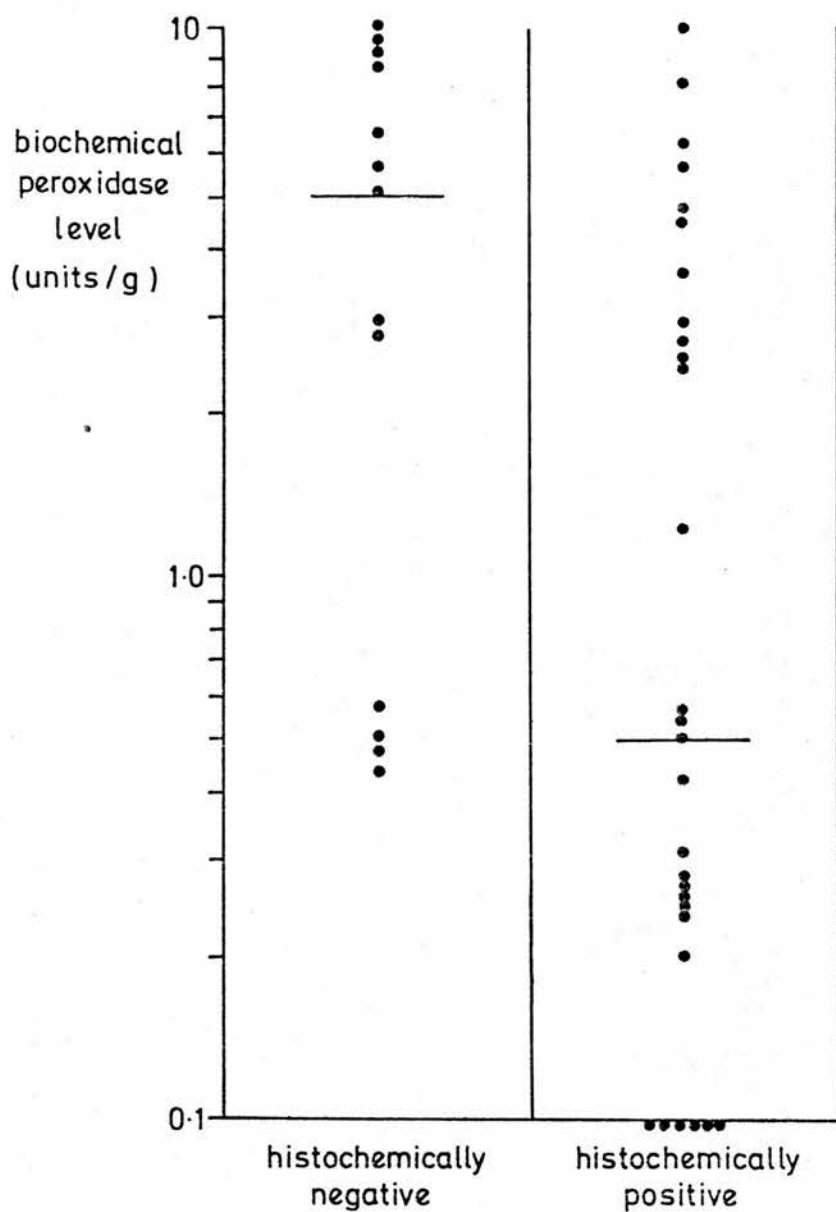


Figure 19.

Biochemical estimates of peroxidase activity in 13 tumours designated histochemically peroxidase-negative and 29 tumours designated histochemically peroxidase-positive.

Each symbol represents the value for a single tumour. Bars indicate the median values.

$p < 0.01$ . (Wilcoxon Rank Sum Test).

VI. PRELIMINARY INVESTIGATIONS INTO THE SITES AND  
MAGNITUDE OF LOSSES OF E/R ACTIVITY DURING  
HISTOCHEMICAL PROCESSING

Because the E/R protein is known to be water-soluble and thermolabile, it was felt that the "rigours" of histochemical processing might result in loss of E/R activity. In particular, cellular disruption during the cutting of frozen sections and exposure to aqueous processing media might permit the leaching-out of the soluble E/R from the tissue into the media; also, exposure of the fragile E/R molecule to conventional histological fixatives might denature the protein, resulting in loss of activity. Preliminary experiments were undertaken to quantitate the effects of these stages of histochemical techniques on E/R activity. Such activity was assessed by a biochemical DCC assay on tissue exposed to each histochemical step.

A. Frozen Section Cutting

An E/R-rich tissue (rat uterus) was removed from the host animal and a 20 mg portion homogenised and assayed for E/R by the standard DCC method. (In view of the small volume of cytosol produced, only three concentrations of  $^3\text{O}_2$  were employed on this occasion.) A second 20 mg portion of the uterus was divided into 4  $\mu\text{m}$  frozen sections which were collected in a cold "Luckham" tube. The sections were homogenised and assayed for E/R in the same way as the "intact" portion.

RESULTS

Scatchard plots of the data from E/R assays on the "intact" and "sectioned" portions of uterus are reproduced in Figure 20. The concentration of E/R ( $P_0$ ) in the "intact"



and "sectioned" portions of uterus are reproduced in Figure 20. The concentration of E/R ( $P_o$ ) in the "intact" portion was 6.69 fmoles/mg wet weight compared with only 2.45 fmoles/mg in the "sectioned" portion.

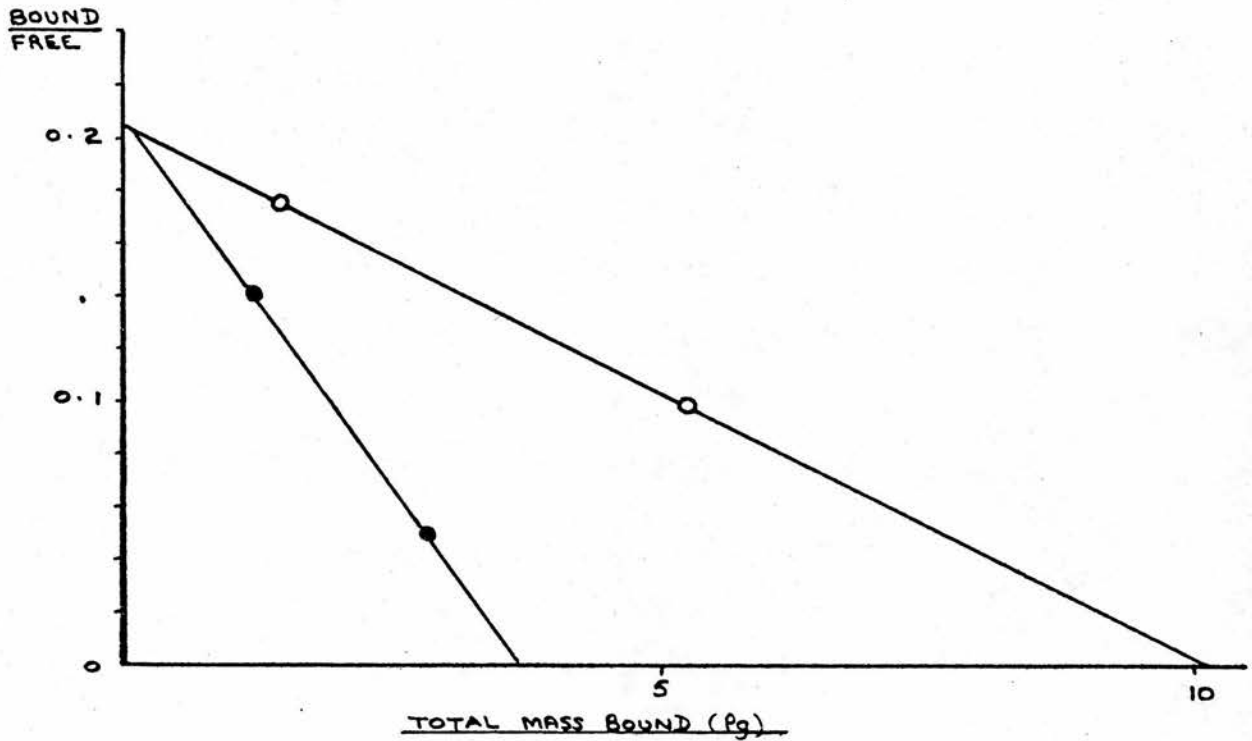


Figure 20.

Scatchard plots of E/R assay data on 20 mg. portions of rat uterus.

o = Intact portion.

● = Portion cut into 4 $\mu$ m. frozen sections.

## B. Exposure To Aqueous Processing Media

A portion of an E/R-rich tissue (DMBA-induced, rat mammary tumour) weighing approximately 250 mg was divided into 4  $\mu$ m cryostat sections which were collected in a cold "Luckham" tube. Tris buffer (2 ml) was then added to the tube and the contents mixed gently to simulate the exposure of frozen-sections to aqueous media during histochemical processing. The sections and buffer were then separated by centrifuging at 1500 rpm for 10 min. The buffer was removed with a Pasteur pipette and assayed for E/R by the standard DCC method. A cytosol was prepared from the sections and also assayed for E/R. The experiment was repeated using 14  $\mu$ m rather than 4  $\mu$ m sections.

## RESULTS

Scatchard plots of the data from E/R assays on the 4  $\mu$ m and 14  $\mu$ m frozen sections and from their respective "processing media" are reproduced in Figure 21. In the case of 4  $\mu$ m sections, four times as much E/R was detectable in the "processing medium" as in the sections. In the case of 14  $\mu$ m sections, there was three times as much E/R activity in the medium as in the sections. In other words, as much as 66 - 75% of the total E/R activity in 14  $\mu$ m or 4  $\mu$ m sections seemed to "leach-out" into aqueous media.

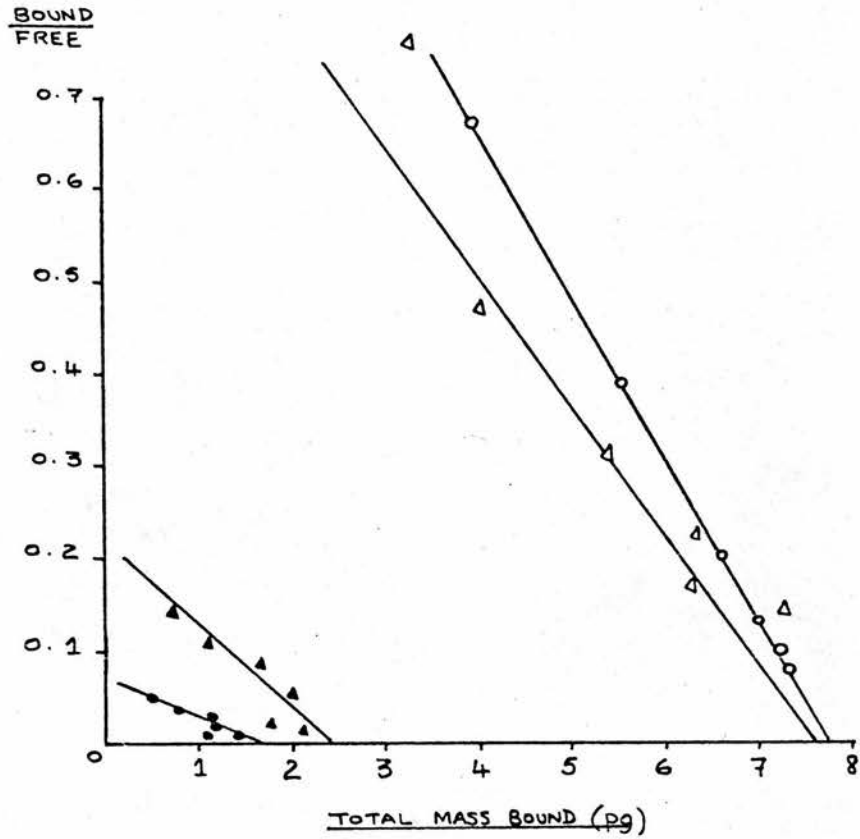


Figure 21.

Scatchard plots of E/R assay data on "processing media" and frozen sections of DMBA-induced rat mammary tumour.

o = Processing medium from 4 $\mu$ m. sections.

● = Cytosol from 4 $\mu$ m. sections.

△ = Processing medium from 14 $\mu$ m. sections.

▲ = Cytosol from 14 $\mu$ m. sections.

### C. Acetone-Fixation

Fixation of frozen sections in acetone prior to incubation with labelled steroid is a component of at least one reported technique of E/R histochemistry (Walker et al., 1980). Preliminary experiments were undertaken to quantitate the effect of such a fixation step on the E/R activity present.

#### a) E/R in the cytosols prepared from acetone-fixed tissues.

This experiment was performed in triplicate. On each occasion, rat uteri, weighing approximately 500 mg in total, were harvested from the host animals. Each uterus was bisected and one horn allocated to "control-" and the other to "acetone-" treatments. The uterine horns were snipped into small fragments and those allocated to acetone-treatment were immersed in acetone at 4°C for 4 min. The "control" fragments were immersed in Tris buffer at 4°C for 4 min. Both sets of uterine fragments were then rinsed in several changes of Tris buffer. After rinsing, cytosols were prepared from each set of fragments and assayed for E/R by the standard DCC method.

### RESULTS

Scatchard plots of the data from E/R assays on cytosols from each of the three acetone-treated tissues and each of the three control tissues are reproduced in Figure 22. In all three instances, the E/R activity detectable in acetone-treated tissue was considerably less than in the control tissue.

#### b) E/R in the insoluble, tissue pellets prepared from acetone-fixed tissues.

As in experiment a), rat uterine horns were allocated

SCATCHARD PLOTS OF E/R ASSAY ON 3 RAT UTERI TO SHOW EFFECT OF ACETONE FIXATION

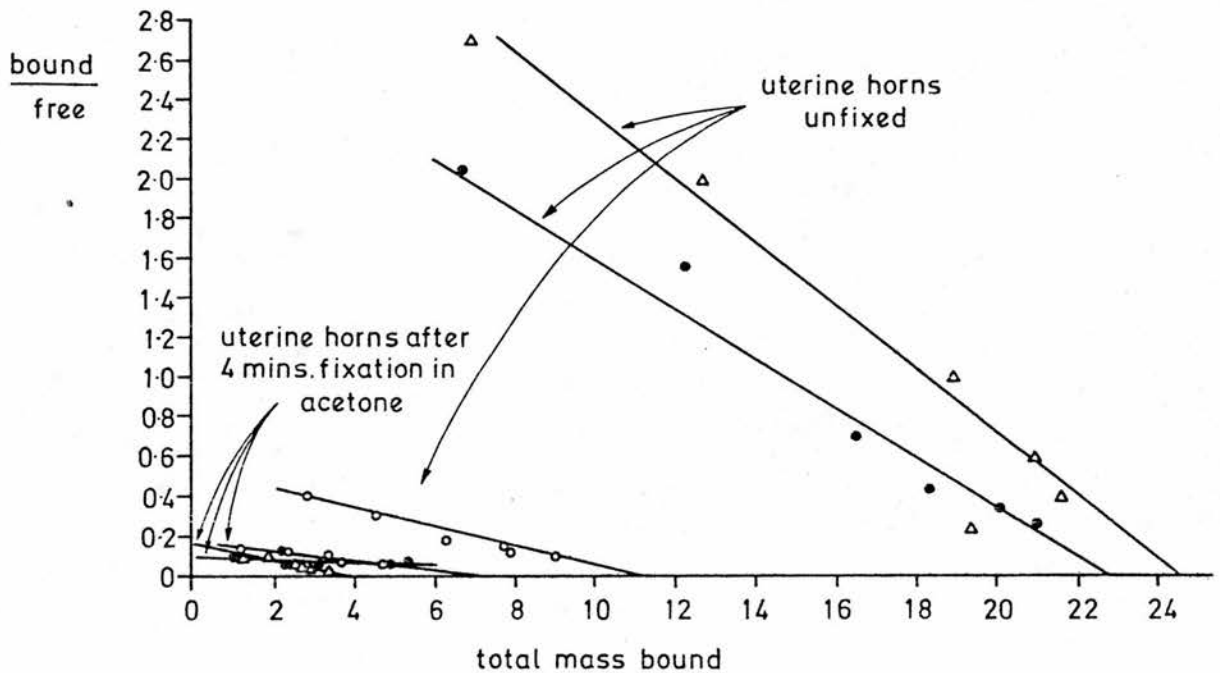


Figure 22.

Scatchard plots of E/R assay data from cytosols prepared from rat uterine horns, fixed in acetone or unfixed.

The plots represented by the same symbol represent portions of the same uterus.

to "acetone" or "control" treatments. The appropriately-treated, uterine fragments were again washed, homogenised and centrifuged and the resulting cytosols were assayed for E/R activity. In addition, the centrifugation pellets were resuspended in Tris buffer (equivalent to 300 mg of original tissue weight per ml) and 200  $\mu$ l aliquots were subjected to E/R assay by the solid-phase system (Method 2).

### RESULTS

Inspection of the raw data revealed that there was no E/R activity present in the pellet preparation from the control (unfixed) uterine horns. The raw data from the assays on the "acetone-treated" pellet, "acetone-treated" cytosol and "control" cytosol revealed E/R activity in all 3 samples. Scatchard plots were drawn and are reproduced in Figure 23. E/R activity was demonstrable in both pellet and cytosol after acetone-treatment, but both concentration ( $P_0$ ), and dissociation constant ( $K_d$ ), were markedly diminished. The total E/R concentration (pellet plus cytosol) detectable in the acetone-treated tissue was less than half of that detectable in the cytosol from the untreated tissue.



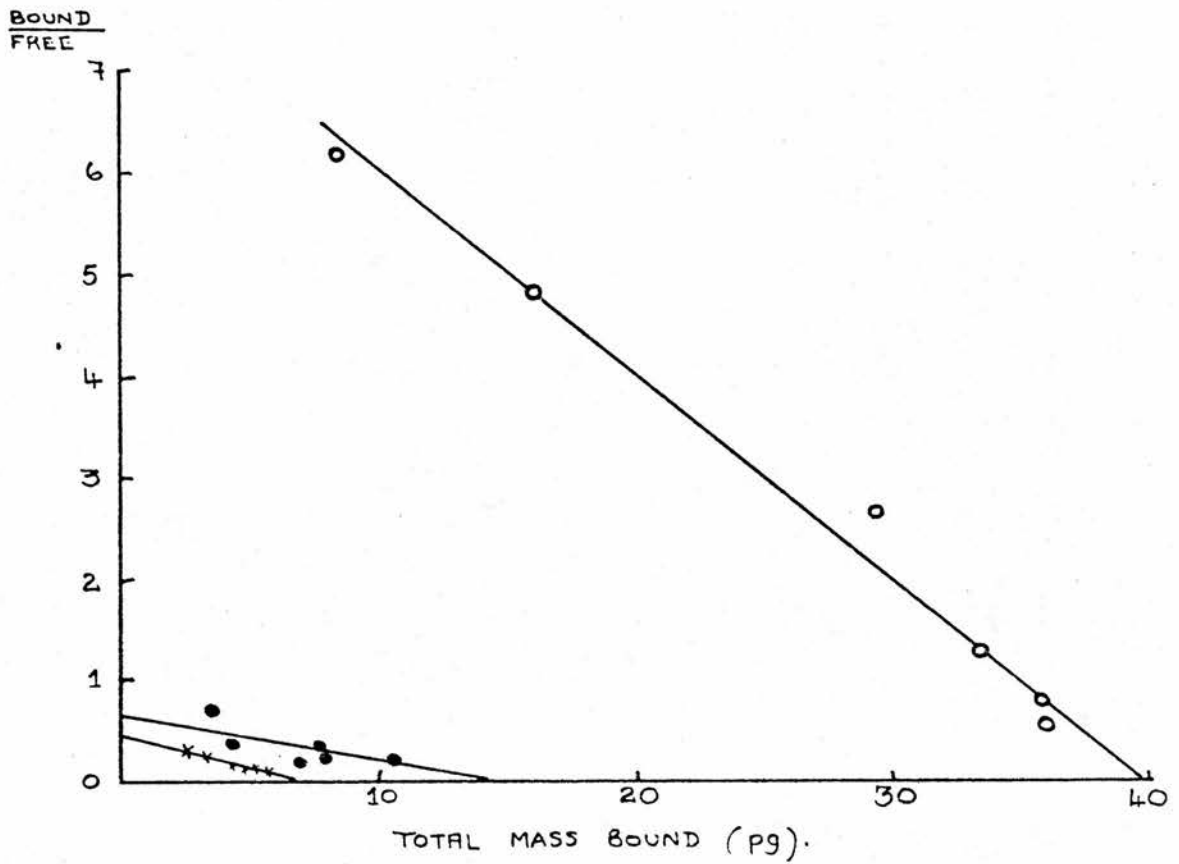


Figure 23.

Scatchard plots of E/R assay data on preparations from rat uteri, fixed in acetone or unfixed.

o = Cytosol assay on unfixed tissue.

x = Cytosol assay on acetone-fixed tissue.

● = Pellet assay on acetone-fixed tissue.

(No E/R activity was detectable in pellet assay on unfixed tissue.)

Results of cytosol and pellet assays are not directly comparable as reagent concentrations were not identical.

D. Ethanol/Acetone "Post-Fixation".

Another approach to the immobilisation of E/R in tissue sections is that described by Pertschuk et al. (1979). Unfixed, frozen sections are incubated with fluorescein-labelled oestradiol, rinsed briefly, then fixed for 10 min in a mixture of equal volumes of ethanol and acetone. The sections are then extensively washed and mounted for fluorescence microscopy. An experiment was undertaken to investigate the effect of a fixation procedure of this kind on E/R activity. Such activity was assessed by determination of the saturable binding of [ $^3\text{H}$ ]  $\text{E}_2$  under conditions similar to those previously used in this department to study nuclear translocation of E/R.

An E/R-rich tissue (DMBA-induced, rat, mammary tumour) was removed from the host animal and cut into slices 1 mm thick. Each slice weighed approximately 50 mg. Two such slices were placed into each of 8 small, conical flasks. To 4 of the flasks was added a solution of [ $^3\text{H}$ ]  $\text{E}_2$  (equivalent to 40,000 cpm; ) in 2 ml of Tris buffer. A similar solution, to which had been added 50,000 pg of  $^0\text{E}_2$ , was added to each of the other 4 flasks. All the slices were allowed to incubate for 1 hour at 25°C, with gentle agitation.

The slices from 2 of the flasks containing [ $^3\text{H}$ ]  $\text{E}_2$  alone and from 2 of those containing [ $^3\text{H}$ ]  $\text{E}_2$  plus  $^0\text{E}_2$  were then briefly rinsed and immersed in ethanol/acetone for 10 min to simulate the histochemical, "post-fixation" process. All slices were then transferred to clean flasks and allowed to wash in Tris buffer to remove steroid not bound to the tissue. The slices from each flask were then transferred to a counting vial, weighed, digested in 5N sodium hydroxide, mixed with "aqueous" scintillator fluid and the uptake of tritium-labelled steroid by each set of slices was determined.

RESULTS

The uptake of tritium by slices incubated with [ $^3\text{H}$ ]  $\text{E}_2$  plus or minus excess  $^0\text{E}_2$  are presented in Table 7. In the "unfixed" slices, marked tritium-labelling occurred in those incubated with [ $^3\text{H}$ ]  $\text{E}_2$  alone and such labelling was considerably diminished in those co-incubated with  $^0\text{E}_2$ . Such binding of labelled  $\text{E}_2$  which could be blocked by the presence of excess, unlabelled hormone was taken as indicating the presence of E/R activity. In the case of slices which were "post-fixed" in ethanol/acetone, minimal labelling with [ $^3\text{H}$ ]  $\text{E}_2$  was detectable. This suggested that any binding of steroid to tissue which occurred during the incubation period had been abolished by exposure to the fixative.

Table 7.

Tritium-labelling of tissue sections with and without ethanol/acetone post-fixation treatment.

FIXATION	INCUBATION MEDIUM	COUNTS BOUND (cpm/mg.tissue).
NONE	$(^3\text{H})\text{E}_2$ only	183
"	$(^3\text{H})\text{E}_2 + ^0\text{E}_2$	51
ETHANOL/ACETONE	$(^3\text{H})\text{E}_2$ only	4
"	$(^3\text{H})\text{E}_2 + ^0\text{E}_2$	6

Each result represents the mean from 2 similarly-treated flasks of sections.

## VII. INVESTIGATIONS INTO POSSIBLE METHODS OF REDUCING LOSS OF E/R ACTIVITY DURING HISTOCHEMICAL PROCESSING

### A. Ammonium Sulphate Precipitation

The ability of ammonium sulphate solutions to precipitate serum proteins is well documented (Cohn et al., 1940). An experiment was undertaken to determine whether or not ammonium sulphate could be used to precipitate the E/R protein in tissue sections, thus reducing "leaching-out" into aqueous media during histochemical processing while avoiding the inactivation associated with conventional methods of tissue fixation.

An E/R-rich tissue (DMBA-induced rat mammary tumour) was divided into 4 x 200 mg portions. Each portion was cut into 4  $\mu$ m cryostat sections and the sections from each portion were collected in a cold "Luckham" tube. To each of the 4 tubes was added 2 ml of one of the following solutions: plain Tris buffer or Tris buffer containing ammonium sulphate to 25, 50 or 75% of saturation. The tubes were gently mixed to simulate the exposure of frozen sections to processing-media during histochemical techniques. The "processing-media" and sections were then separated and each was assayed for E/R activity as described in the preliminary experiment on p110.

## RESULTS

Scatchard plots of the data from E/R assays on each of the 4 sets of frozen sections and each of the 4 "processing-media" are shown in Figure 24. The ratio of E/R activity detectable in the "processing medium" against that detectable in the frozen sections for each concentration of ammonium sulphate is shown in Table 8. The proportion of the total E/R activity which "leached-out" into the medium decreased

with increasing concentration of ammonium sulphate. The Scatchard plots for 75% saturated solution suggest, however, that the total, detectable E/R activity was diminished by exposure to this concentration of ammonium sulphate.

EFFECT OF INCREASING CONCENTRATION OF  $(\text{NH}_4)_2\text{SO}_4$  ON "LEACHING-OUT" OF ER INTO WASH BUFFER.

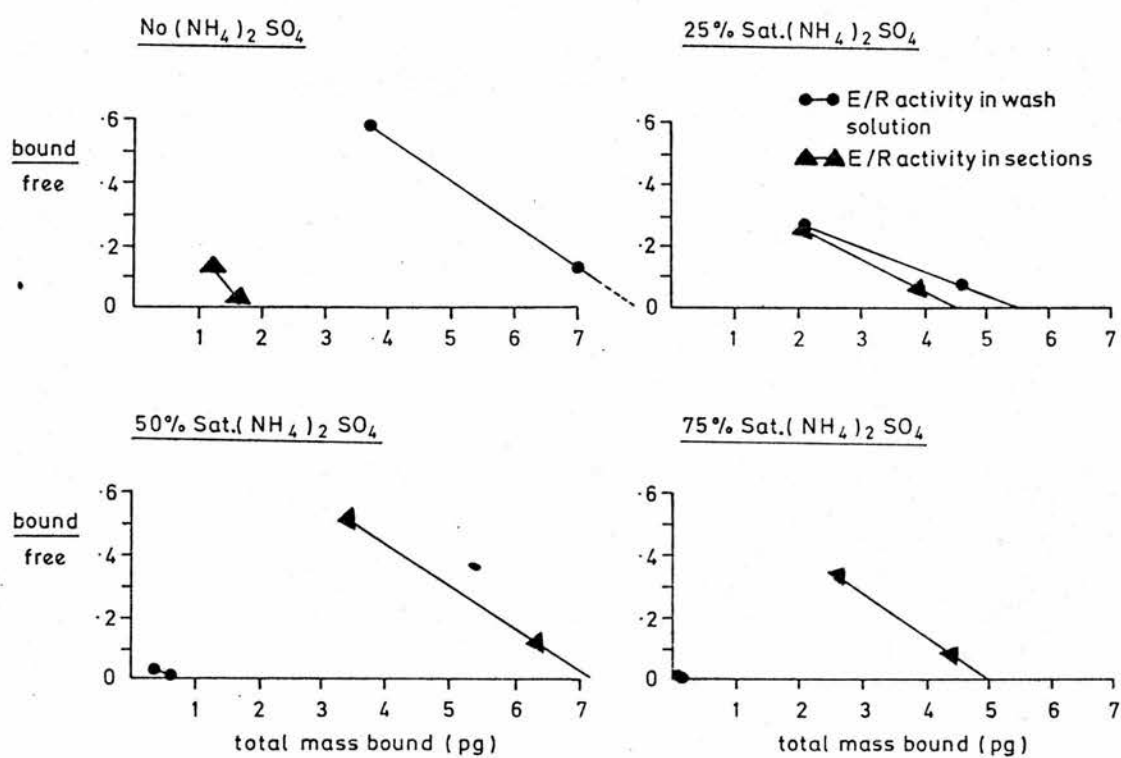


Figure 24.

Scatchard plots of E/R assay data on "processing media" and frozen sections of DMBA-induced rat mammary tumour.

The media contained various concentrations of ammonium sulphate.



Table 8.

The effect of various concentrations of ammonium sulphate on the "leaching-out" of E/R activity from frozen sections into processing media.

% saturation $(\text{NH}_4)_2\text{SO}_4$ .	Ratio E/R activity in <u>medium</u> sections
0	4.6/1
25	1.2/1
50	0.1/1
75	0.05/1

## B. Protamine Sulphate Precipitation

Protamine sulphate, like ammonium sulphate, induces precipitation of various proteins, including E/R. It was felt that treatment of frozen sections with protamine sulphate might provide a means of immobilising E/R without inactivation. As a preliminary investigation into the feasibility of such an approach, an attempt was made to reproduce the results of Chamness et al. (1975) and of Lippman and Huff (1976) in demonstrating the retention of E/R activity in protamine-precipitated pellets prepared from an E/R-rich cytosol. A cytosol was prepared from rat uteri and aliquots were assayed for E/R by the standard DCC method (Method 1). Further aliquots were subjected to the "solid-phase" assay technique (Method 2)\*. The experiment was performed in duplicate.

## RESULTS

Scatchard plots of the data from the E/R assays on the cytosol and protamine-precipitate from each of two rat uteri are reproduced in Figure 25. The values of  $P_0$  and  $K_d$  from each assay are presented in Table 9.

Similar values of  $P_0$  (concentration of E/R) for each uterine specimen were derived from the cytosol and solid-phase assays. The  $K_d$  (reflecting strength of E/R binding) was, in contrast, apparently impaired in the solid-phase preparations of both specimens.

\* where the solid - phase pellets were prepared in a final concentration of 0.75 mg/ml. protamine sulphate.

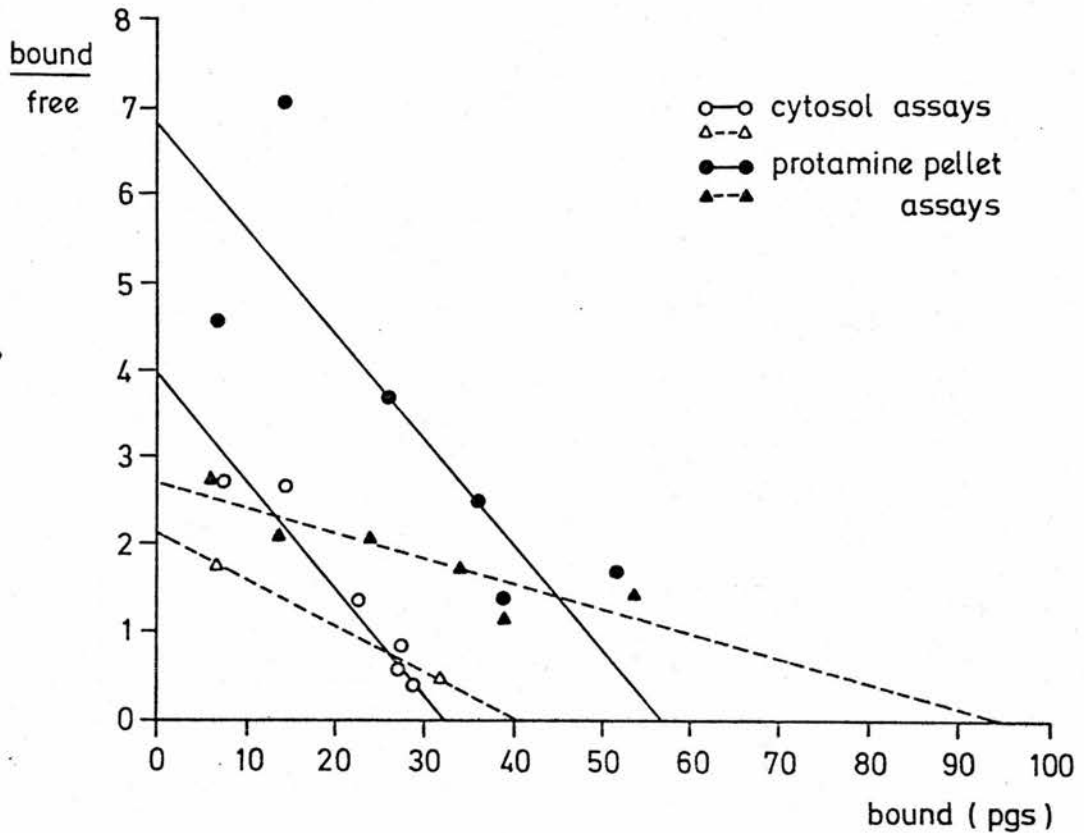


Figure 25.

Scatchard plots of E/R assay data on preparations from rat uteri.

○ = Cytosol assay. Specimen 1.

△ = Cytosol assay. Specimen 2.

● = Protamine pellet assay. Specimen 1.

▲ = Protamine pellet assay. Specimen 2.

(Note that pellet assays were undertaken on tissue aliquots of double the mass of the aliquots used for cytosol assays and, thus, Scatchards are not directly comparable.)

Table 9.

Characteristics of E/R activity detectable in cytosols and protamine sulphate precipitated pellets from two specimens of rat uterus.

PREPARATION	$P_0$	$K_d$
CYTOSOL 1.	15.4	$0.31 \times 10^{-10} \text{ M.}$
CYTOSOL 2.	11.4	$0.85 \times 10^{-10} \text{ M.}$
PELLET 1.	15.5	$5.2 \times 10^{-10} \text{ M.}$
PELLET 2.	10.1	$1.4 \times 10^{-10} \text{ M.}$

# VIII. EVALUATION OF HISTOCHEMICAL TECHNIQUES USING GLASS - MOUNTED FROZEN SECTIONS

In the experiments described in the preceeding sections the E/R activity remaining in tissues after processes analogous to those used in histochemistry has been quantitated by means of conventional E/R assays. In the following experiments an attempt has been made to evaluate the effects of similar processes on the E/R which can be detected in glass-mounted frozen sections, a situation which more closely resembles the proposed histochemical methodology.

## A. E/R Activity In Unfixed, Glass-Mounted Frozen Sections

This experiment was performed on 5 occasions. In each case, portions of the tissue under study were cut into cryostat sections, mounted on coverslips, incubated with [ $^3\text{H}$ ]  $\text{E}_2$  with or without  $^0\text{E}_2$ , washed for varying periods of time in buffer and tritium-labelling determined as described in "Method 3".

## RESULTS

The results of these studies in E/R-rich tissues (rat uterus and DMBA-induced mammary tumours) and in an E/R-negative tissue (rat cerebral cortex) are presented in Table 10. Both 4  $\mu\text{m}$  and 14  $\mu\text{m}$  sections, and both minimal and extensive washing regimes, were studied. After minimal washing, there was enormous tritium-labelling of the sections which was not diminished by co-incubation with  $^0\text{E}_2$ . In the E/R-rich tissues subjected to a 1 hour washing process, differential labelling between the sections incubated with [ $^3\text{H}$ ]  $\text{E}_2$  alone and those co-incubated with  $^0\text{E}_2$  occurred, this is indicative of E/R activity. In the case

of the E/R-negative tissue studied, similar differential labelling did not occur, although high "non-specific" binding was present.

Table 10.

Tritium-labelling of unfixed, glass-mounted, frozen sections  
after incubation with  $(^3\text{H})\text{E}_2 \pm ^0\text{E}_2$ .

TISSUE	Thickness ( $\mu\text{m.}$ )	Wash time. (hr.)	cpm. $(^3\text{H})\text{E}_2$	cpm. $(^3\text{H})\text{E}_2 + ^0\text{E}_2$
Rat uterus	4	0	115450	152100
DMBA-tumour 1.	4	1	506	120
" 2.	4	1	2223	1505
" 3.	14	1	2534	1948
rat cerebrum	14	1	2125	4442

B. E/R Activity In Acetone-Fixed, Glass-Mounted,  
Frozen Sections

Five tissues were examined for their capacity to bind oestrogen "specifically", after acetone fixation: one human uterus, two DMBA-induced, rat, mammary tumours (E/R-rich tissues), and two samples of rat duodenum (E/R-negative tissue). In each experiment, approximately 200 mg of tissue was cut into frozen sections, mounted onto coverslips and then immersed in acetone, at room temperature, for 4 minutes. The acetone was allowed to evaporate before "flooding" the sections with steroid solutions and processing by the method described for the assay of E/R activity in glass-mounted, frozen sections (Method 3). Post-incubation washing was in three changes of PBS for a total of 1 hour.

RESULTS

The tritium-labelling of the fixed sections from each tissue after incubation with  $[^3\text{H}] \text{E}_2$ , with or without excess  $^0\text{E}_2$ , is shown in Table 11. In each of the "E/R-rich" tissues there was differential labelling between the sections incubated with  $[^3\text{H}] \text{E}_2$  alone and those co-incubated with excess  $^0\text{E}_2$ , indicative of E/R activity. Such differential labelling was not apparent in the case of the E/R-negative tissues.



Table 11.

Tritium-labelling of acetone-fixed, glass-mounted, frozen sections after incubation with  $^3\text{(H)}\text{E}_2 + ^0\text{E}_2$ .

TISSUE	cpm. $^3\text{(H)}\text{E}_2$ only.	cpm. $^3\text{(H)}\text{E}_2 + ^0\text{E}_2$ .
Human uterus	1699	241
DMBA-tumour 1.	1578	656
DMBA-tumour 2.	1048	573
Rat duodenum 1.	238	244
Rat duodenum 2.	207	185

C. E/R Activity In Ammonium Sulphate-Treated, Glass-Mounted, Frozen Sections

This experiment was performed on two occasions using 14  $\mu$ m sections of a DMBA-induced, rat, mammary tumour. On each occasion, one set of frozen sections was subjected to the "glass-mounted E/R assay" (Method 3), without any form of fixation and using Tris buffer as the post-incubation, washing medium. A second set of frozen sections from each tumour was assayed similarly except that the incubation and washing media contained ammonium sulphate at 50% of saturation.

RESULTS

The tritium-labelling of sections incubated with [ $^3\text{H}$ ]  $\text{E}_2$ , with and without excess  $^0\text{E}_2$ , in the presence or absence of ammonium sulphate, is shown in Table 12. E/R activity, indicated by differential labelling between sections incubated with [ $^3\text{H}$ ]  $\text{E}_2$  alone and those co-incubated with excess  $^0\text{E}_2$ , was not convincingly demonstrated in tissues processed in the presence or absence of ammonium sulphate in these experiments. The presence of ammonium sulphate in the processing media seemed to greatly increase "non-specific" binding of tritium-labelled steroid to the tissue sections.

Table 12.

Tritium-labelling of glass-mounted, frozen sections with and without ammonium sulphate in the processing media.

EXPERIMENT	MEDIA	cpm. ( $^3\text{H}$ )E <sub>2</sub> only.	cpm. ( $^3\text{H}$ )E <sub>2</sub> + $^0\text{E}_2$ .
1	no (NH <sub>4</sub> )SO <sub>4</sub>	481	731
1	with (NH <sub>4</sub> )SO <sub>4</sub>	11359	10523
2	no (NH <sub>4</sub> )SO <sub>4</sub>	479	305
2	with (NH <sub>4</sub> )SO <sub>4</sub>	8198	10505

D. E/R Activity In Protamine Sulphate-Treated, Glass-Mounted, Frozen Sections

Frozen sections (14  $\mu$ m) of DMBA-induced, rat, mammary tumour were cut and mounted on glass coverslips. One set of such sections was assayed, unfixed, for E/R activity by Method 3. A second set was "flooded" with a solution of protamine sulphate (1.5 mg/ml) for 5 minutes, then rinsed in PBS prior to incubation with steroid solutions and subsequent processing as described in Method 3.

RESULTS

The tritium-uptake of sections processed with and without protamine-treatment are shown in Table 13. The frozen sections processed in the absence of protamine sulphate-treatment did not show any evidence of E/R activity as indicated by decreased uptake of tritium in the presence of excess  $^0E_2$ . The protamine-treated sections, however, did show differential tritium-labelling between those incubated with [ $^3H$ ]  $E_2$  alone and those co-incubated with excess  $^0E_2$ . However, almost 50% of the total [ $^3H$ ]  $E_2$  binding appeared to be "non-specific", i.e. was not blocked by  $^0E_2$ .

Table 13.

Tritium-labelling of glass-mounted, frozen sections with and without protamine sulphate treatment.

MEDIUM	cpm. ( $^3\text{H}$ )E <sub>2</sub> only.	cpm. ( $^3\text{H}$ )E <sub>2</sub> + $^0\text{E}_2$ .
No protamine.	885	1023
+ protamine.	2071	1070

IX. CHARACTERISATION OF THE CONJUGATES SYNTHESISED FOR  
E/R HISTOCHEMISTRY

Conjugates of oestradiol, BSA and a visible tracer (fluorescein or horseradish peroxidase) were synthesised as described in Methods 7 and 8. The following investigations were made in an attempt to confirm the incorporation of all three components into the final conjugates.

A. Fluorescein Conjugates

Pertschuk et al. (1980) reported that they were able to confirm incorporation of oestradiol, BSA and fluorescein into their conjugates by the demonstration of absorbance peaks at 279, 280 and 495 mμ. respectively. The absorbance spectra of samples of oestradiol-BSA-fluorescein donated by Dr. Pertschuk and synthesised by the present author and also of the BSA-fluorescein "control" conjugate synthesised by this author were constructed using a Unicam SP800 U.V. scanning spectrophotometer.

RESULTS

The absorbance spectra of the three conjugates, together with the spectrum of a reference sample of albumin solution, are reproduced in Figure 26. Consideration of the fluorescein peaks at 495 mμ. indicates that incorporation of fluorescein into the author's conjugate was similar to that found in Dr. Pertschuk's. It proved impossible, in practice, to distinguish between the peaks for oestradiol and BSA at 279 and 280 mμ. respectively. In this region of the absorbance spectra, therefore, no distinct difference could be discerned between the absorbance patterns of conjugates containing both oestradiol and BSA and that

containing BSA but no oestradiol. These spectra do, however, indicate that the "control" conjugate synthesised by the author is less heavily labelled with fluorescein per mg of conjugate than either of the oestradiol-containing conjugates.

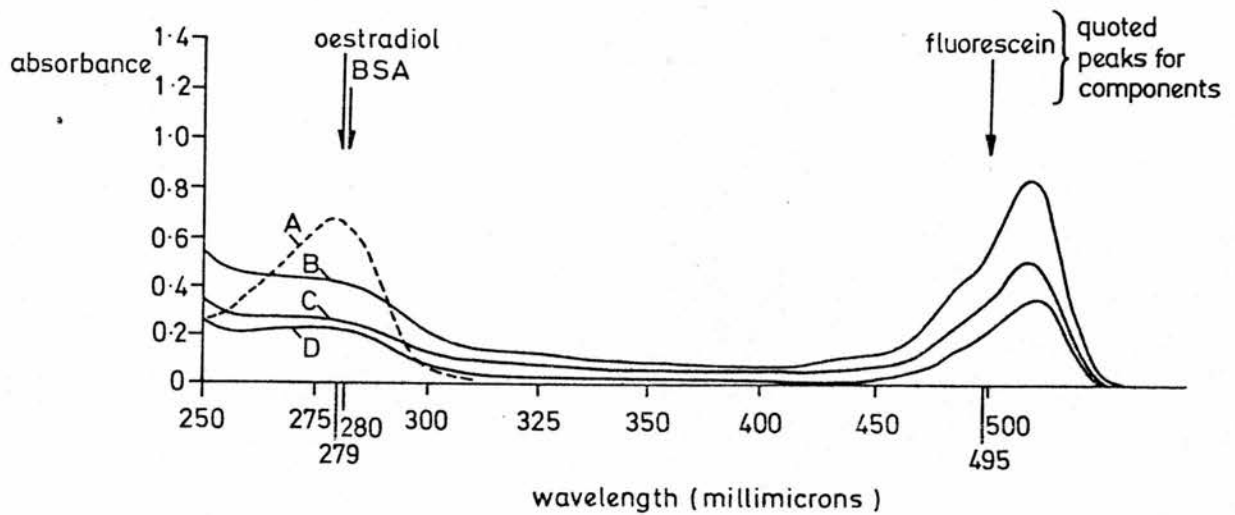


Figure 26.

Absorption spectra of fluoresceinated conjugates.

A = Albumin reference curve.

B = Oestradiol-BSA-Fluorescein synthesised by the author.  
(0.1 mg/ml.)

C = Similar conjugate synthesised by Dr. L.Pertschuk.  
(0.05 mg/ml.)

D = BSA-Fluorescein. (0.1 mg/ml.)



## B. Peroxidase Conjugates

The second class of conjugates studied comprised oestradiol, BSA and peroxidase and were based on that described by Walker et al. (1980). For the synthesis of these conjugates a commercial preparation of BSA-oestradiol (from Steroids) was purchased and the final conjugation step, the linkage of HRP, was undertaken by the author, as described in Method 8. An aliquot of the final conjugate solution (Method 8a) was assayed for peroxidase activity by the routine guaiacol method (Method 5) in order to confirm the incorporation of active peroxidase into the final material.

## RESULTS

The peroxidase activity of the final conjugate was found to be 40 units/ml, indicating that the final conjugate was rich in active peroxidase.

## X. E/R - BINDING AFFINITIES OF OESTRADIOL CONJUGATES

The following experiments were undertaken in an attempt to confirm that the oestradiol conjugates, which had been synthesised for use in E/R histochemistry, were, in fact, able to bind to E/R, at least in its cytosolic form. In addition, the relative binding affinities of these conjugates for the E/R (relative to that of oestradiol-17 $\beta$ ) were determined to aid in the selection of appropriate conjugate concentrations for histochemical techniques. The ability of the compound under test to displace [ $^3\text{H}$ ]  $\text{E}_2$  from cytosol binding sites was regarded as evidence of E/R binding. The following compounds were subjected to competitive binding studies:

1. Oestradiol 17 $\beta$ .
2. Oestradiol - BSA - fluorescein donated by Dr. L. Pertschuk.
3. Oestradiol - BSA - fluorescein synthesised by the author.
4. BSA - fluorescein "control" conjugate.
5. 6 keto - oestradiol 17 $\beta$  (precursor of the peroxidase conjugate).
6. Oestradiol - BSA (intermediate in preparation of peroxidase conjugate).
7. Oestradiol - BSA - peroxidase.
8. CI628 (anti-oestrogen used as an E/R "blocker").

For these studies, aliquots (100  $\mu\text{l}$ ) of an E/R-rich cytosol, prepared from pooled rat uteri, were incubated with a fixed mass (10 pg) of [ $^3\text{H}$ ]  $\text{E}_2$  and varying masses of the test compound, in a manner similar to that described for the routine assay of E/R activity.

## RESULTS

The increasing displacement of [ $^3\text{H}$ ]  $\text{E}_2$  from cytosolic receptor sites by increasing concentrations of the various test compounds is shown in Figure 27 (fluorescein conjugates and CI628) and Figure 28 (peroxidase conjugate and precursors). A curve for oestradiol-17 $\beta$  is shown in each case for comparison. With the exception of the "control" conjugate, BSA - fluorescein, all the compounds tested were able to displace [ $^3\text{H}$ ]  $\text{E}_2$  from E/R binding sites. The binding affinities of the compounds studied varied considerably as indicated by the variations in molar concentration needed to achieve 50% displacement of [ $^3\text{H}$ ]  $\text{E}_2$ . The ratio of the concentration needed for oestradiol-17 $\beta$  to that needed for each test compound, (i.e. the relative binding affinity) is presented in Table 14. The affinities are expressed in terms of the binding potency of 1 mole of each conjugate (containing between 5 and 33 moles of oestradiol) or steroid relative to a value of 1 for oestradiol-17 $\beta$ .

Under the conditions of this study, CI628 was found to have the highest affinity for E/R binding sites, being 4 times as potent as oestradiol-17 $\beta$ . The precursor of the peroxidase conjugate, 6 keto-oestradiol 17 $\beta$ , had a relative potency of only 0.13 and the conjugates based on it proved to be less potent than the fluorescein conjugates.

## AFFINITIES OF STEROIDS AND CONJUGATES FOR OESTROGEN RECEPTOR

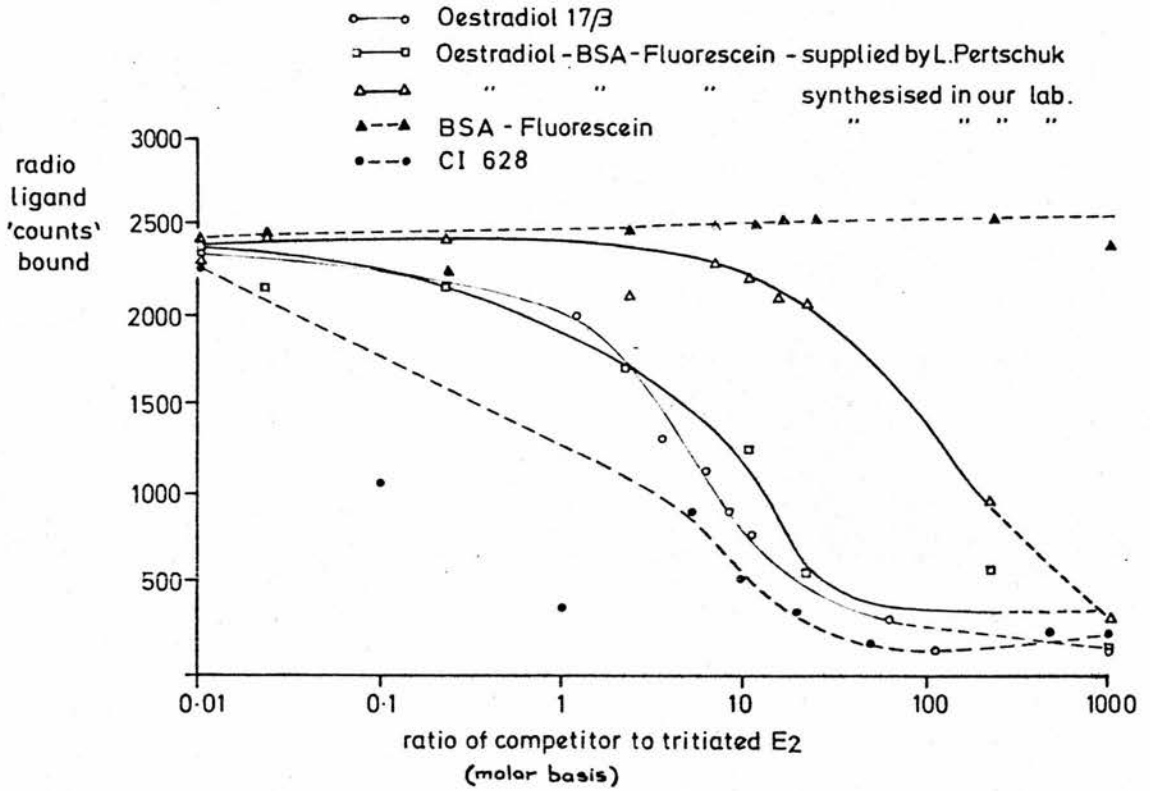


Figure 27.

Curves illustrating the displacement of  $^3\text{H}$ Oestradiol from cytosol receptor sites by fluoresceinated conjugates and related compounds.

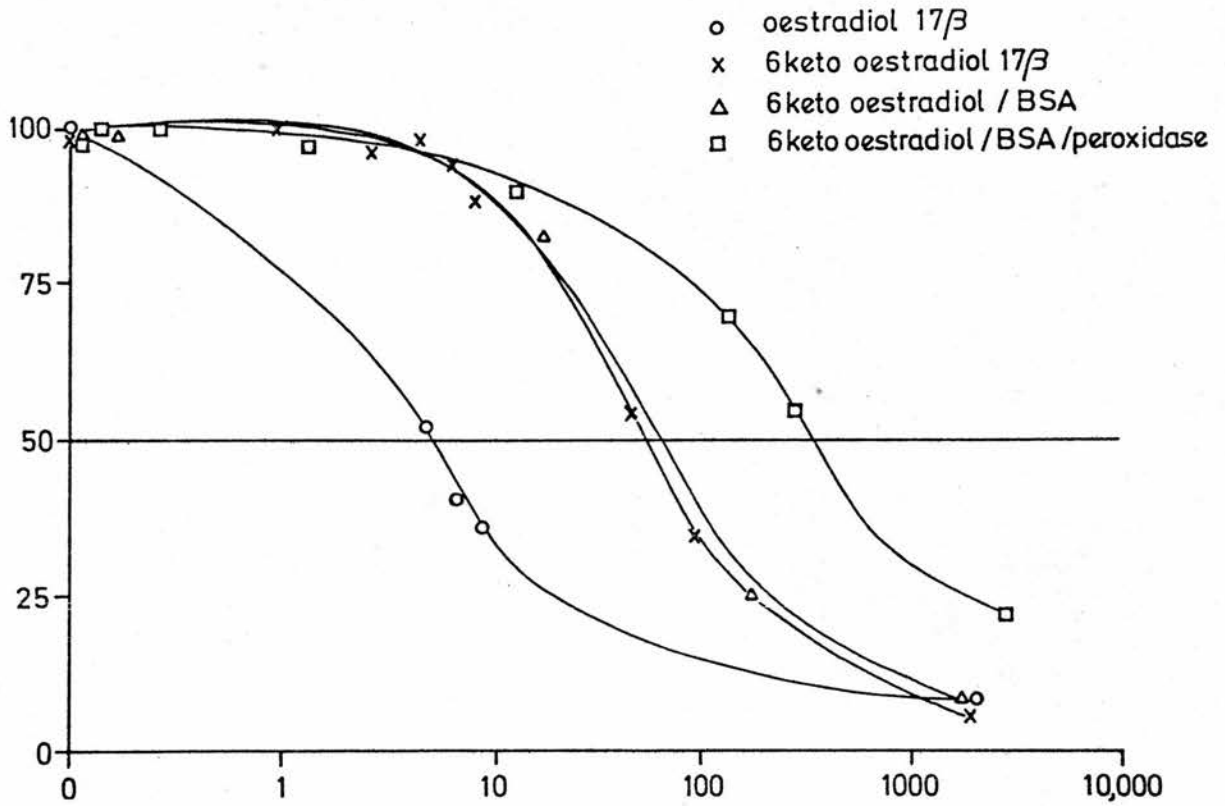


Figure 28.

Curves illustrating the displacement of ( $^3\text{H}$ )Oestradiol from receptor sites by peroxidase conjugate and related compounds.

Figures on the "Y" axis indicate "radio-ligand bound" as a % of the total bound in the absence of competitor.

Figures on the "X" axis indicate the ratio of competitor to ( $^3\text{H}$ )E $_2$  on a molar basis.

Table 14.

Relative binding activities of conjugates, conjugate precursors and control solutions as used for E/R histochemistry. Binding activities relate to the molar concentrations of each compound which causes 50% inhibition of ( $^3\text{H}$ )E<sub>2</sub> binding, on a basis of Oestradiol 17 $\beta$  = 1.

COMPOUND.	Relative activity.
Oestradiol 17 $\beta$	1
Oestradiol-BSA-Fluorescein (Pertschuk).	0.8
" (Present author ).	0.05
BSA-Fluorescein.	0.00
6-keto-oestradiol 17 $\beta$ .	0.13
6-keto-oestradiol-BSA.	0.09
6-keto-oestradiol-BSA-peroxidase.	0.02
CI 628	4.4

XI. EVALUATION OF OESTRADIOL CONJUGATES FOR THE  
LOCALISATION OF E/R IN FROZEN SECTIONS

A. Fluorescein Tracer

The histochemical technique of Pertschuk et al. (1979), described in Method 9, was carried out on a range of human and rat tissues using a specimen of the oestradiol-BSA-fluorescein conjugate donated by Dr. Pertschuk and a specimen of the similar conjugate synthesised by this author (Method 7). The tissues examined included biochemically E/R-rich tissues (DMBA-induced rat mammary tumours and rat uteri), a biochemically E/R-negative tissue (rat duodenum), a tissue reported by Dr. Pertschuk as strongly histochemically E/R-positive (rat skin) and also frozen sections and cytological smears of human mammary cancers.

RESULTS

As a baseline for the interpretation of sections incubated with fluorescein conjugates, fresh sections of rat uterus and duodenum were mounted and examined under the fluorescence microscope and auto-fluorescence was found to be minimal.

Examination of sections of rat uterus incubated with the oestradiol-BSA-fluorescein conjugates revealed widespread fluorescence. In particular, the nuclei of certain cells of the myometrial layer were brightly fluorescent, as was the cytoplasm of the endometrial cells. In control sections incubated with the conjugate of BSA and fluorescein alone, the distribution of fluorescence was similar but the overall intensity was less. In those control sections co-incubated with an excess of competitor (diethylstilboestrol, CI 628 or tamoxifen) no diminution of fluorescence was observed.

Even when the concentration of competitor was increased to the point of saturation, the intensity of fluorescence appeared to be unimpaired, although very high competitor-concentrations appeared to have a non-specific "tissue-disruptive" effect.

Sections of rat duodenum, biochemically an E/R-negative tissue, were incubated with the same three solutions. In those incubated with oestradiol-BSA-fluorescein, a widespread fluorescence, particularly intense in the endothelial layer, was seen. The distribution of fluorescence in a transverse section of duodenum was remarkably similar to that seen in a similar section of uterus. Again, sections of duodenum incubated with the conjugate of BSA and fluorescein alone showed a similar, but less intense fluorescence and those co-incubated with the competitors showed tissue damage but no diminution of fluorescence.

In rat and human mammary tumours, widespread fluorescence was seen in all specimens examined. Again, the appearances of the "control" sections were of a similarly distributed but less intense fluorescence in the case of BSA-fluorescein-incubated sections, and of tissue disruption but unimpaired fluorescence in the case of sections co-incubated with competitors. In the specimen of rat skin, certain cells at the bases of hair follicles exhibited very bright fluorescence but, as with the other tissues studied, this fluorescence was not diminished in sections co-incubated with excess competitor.

In a further experiment, sections of rat uterus were washed in "normal swine serum" prior to incubation with the conjugates in an attempt to block non-specific protein binding sites. However, this treatment did not result in a reduction in the distribution of tissue fluorescence.

It was noted that the appearances obtained after



incubation with Dr. Pertschuk's conjugate were essentially the same as those obtained after incubation with the conjugate synthesised by the author.

## B. Peroxidase Tracer

Human and rat tissues were studied by the method of Walker et al. (1980) using a conjugate of oestradiol, BSA and HRP (Method 10). Conjugates prepared by the method of Nakane and Kawaoi (8A) and by the method of Avrameas and Ternynck (8B) were used. The tissues examined included biochemically E/R-positive tissues (human and rat reproductive tract and human tumours of mammary origin) and biochemically E/R-negative tissues (rat duodenum, skin and muscle and human mammary tumours). Prior to incubation with HRP-labelled conjugates, comparable sections of all the tissues under study were stained for endogenous peroxidase as in Method 6.

## RESULTS

### a) Endogenous Peroxidase

Endogenous peroxidase staining was found to be abundant in the sections of human uterus and rat duodenum examined. These tissues were, therefore, considered unsuitable for study by the peroxidase-tracer technique as it would have been impossible to distinguish between endogenous and HRP staining. In all other tissues examined, endogenous peroxidase staining was minimal or absent (as indicated in the examples, Figures 29 - 32) and did not interfere with the interpretation of sections incubated with peroxidase-labelled steroid.

### b) Concentration Of Conjugate Used

The concentration of each conjugate to be used for histochemical studies was determined by preliminary staining of sections of rat uterus with various dilutions and selecting the one which gave rise to easily visible, but

localised, brown staining. Conjugates GP1 (prepared by Method 8a) and GP4 (prepared by Method 8b) which had not been purified (and hence diluted) by Sephadex chromatography were used at dilutions of 1/8 to 1/20. Conjugate GP3 (prepared by Method 8b) had been diluted during Sephadex chromatography and it was found necessary to use it without further dilution. Conjugate GP2 (Method 8b), which was also purified on Sephadex, was found to be too dilute to produce any detectable staining.

c) Appearances After Histochemical Processing

The microscopic appearances after histochemical processing of 25 tissues, in which interpretation was not complicated by the presence of endogenous peroxidase staining, are summarised in Table 15. The E/R content of each tissue, as measured by DCC assay, is shown for comparison with the histochemical appearances. In all the tissues with high E/R levels, as assessed biochemically, cellular uptake of conjugate was demonstrable. However, when the same tissue was examined on several occasions (e.g. rat uterus, assays 5 - 7 and breast cancer metastasis, assays 21 - 24) reproducibility seemed to be very poor. Cellular uptake of conjugate was also demonstrable in many of the biochemically E/R-negative tissues studied, for example, the squamous epithelium of rat ear, rat cheek muscle and some human tumours (assays 18 and 19).

Attempts were made to demonstrate blocking of cellular uptake of conjugate in comparable frozen sections by co-incubation with excess of a suitable competitor. Competitors used were diethylstilboestrol (DES), tamoxifen and CI 628 as indicated in the table. In order to obtain blocking in any instances, it was found necessary to use the competitors in saturated solution. CI 628 was found to be unsatisfactory as its yellow colour and tissue-disruptive effect made interpretation of frozen sections which had been

exposed to it very difficult (assays 10 and 11). Convincing blocking was achieved with DES in assays 1, 4 and 21 and with tamoxifen in assay 12. Photomicrographs illustrating this blocking in these 4 tissues are reproduced in Figures 29 - 32. In other tissues which exhibited cellular uptake of conjugate, blocking could not be achieved; this was true of both biochemically E/R-positive tissues (assays 2, 3, 5, 15) and biochemically E/R-negative tissues (assays 8, 9, 18, 19). In other cases (assays 6, 7, 10, 11, 14, 24) the appearances of "co-incubated" frozen sections were regarded as equivocal because of tissue-disruption or precipitation of competitor or DAB over the section.

Overall, of the 25 tissues presented here, 19 had appreciable E/R activity as assessed by DCC assay. Of these 19, only the 4 illustrated in Figures 29 - 32 exhibited cellular uptake of conjugate which was diminished by co-incubation with competitor; thus, only in these 4 might it be claimed that E/R had been demonstrated. Of the 6 E/R-negative or very low positive (DCC basis) tissues, only 2 (assays 13 and 25) exhibited minimal uptake of conjugate. The remaining 4 (assays 8, 9, 18, 19) did exhibit uptake of conjugate but blocking was not demonstrable. In only 6 of the 25 tissues, therefore, was full agreement with DCC assay obtained.

TABLE 15.

Summary of results of histochemical staining for E/R, using peroxidase tracer, in 25 tissues with no detectable endogenous peroxidase staining.

The value for E/R content of each tissue obtained at DCC assay ( $P_0$ ) is shown for comparison.

The conjugates used for staining each tissue are listed according to the following code:

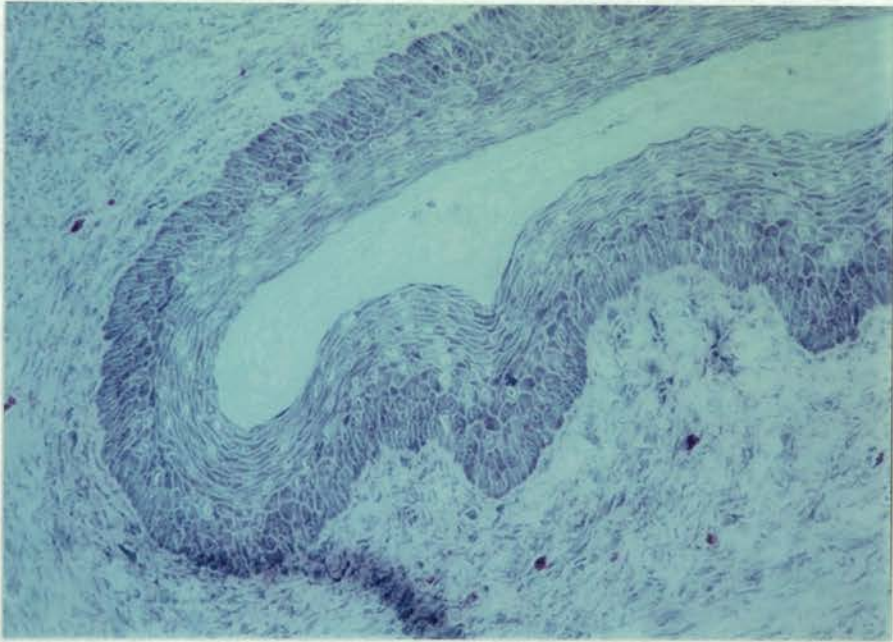
GP1 : conjugate prepared by Method 8a.  
GP3 : " " " " 8b.  
GP4 : second conjugate prepared by method 8b.

---

Assay No.	TISSUE	DCC P <sub>0</sub>	CONJUGATE	BLOCKER	CELLULAR UPTAKE OF CONJUGATE	? BLOCKING	AGREES WITH DCC?
1	Immature rat uterus I	10.24	GP4 1/8	DES	+++ vaginal & ectocervical squamous epithelium.	Partial	+
2	Immature rat uterus II	13.34	GP4 1/12 " 1/16	DES	+++ All layers.	No	?
3	Lactating rat uterus I	14.9	GP4 1/8	DES	+++ endometrium ++ other layers.	No	?
4	Lactating rat uterus I (2nd. assay).	14.9	GP4 1/16	DES	as above.	Partial	?
5	Lactating rat uterus II	11.5	GP4 1/8	DES	+++ all layers.	No	?
6	Lactating rat uterus II (2nd. assay)	11.5	GP4 1/8	DES	+ only.	?	?
7	Lactating rat uterus II (3rd. assay.)	11.5	GP4 1/8	DES	+++ all layers.	?	?
8	Rat ear.	0	GP4 1/8	DES	+++ squamous epith. Partic hair follicles.	No	?
9	Rat cheek muscle.	0	GP4 1/8	DES	++	No	?
10	Human breast cancer. (LD)	3.58	GP1 1/10	CI 628	++ Cancer cells; - stroma & lymphocytes.	?	?
11	Human breast cancer (LD) 2nd. assay.	"	GP1 1/20	CI 628	as above	?	?
12	Human breast cancer (LD) 3rd. assay.	"	GP3	Tamox.	as above	Yes	+
13	Human breast cancer (IM)	0	GP3	Tamox.	none	-	+
14	Human breast cancer (3)	5.0	GP4 1/12 " 1/16	DES	+	?	?
15	Human breast cancer (4)	6.0	GP4 1/8	DES	+	No	?

Assay No.	TISSUE	DCC P <sub>0</sub>	CONJUGATE	BLOCKER	CELLULAR UPTAKE OF CONJUGATE.	? BLOCKING	AGREES W/ DCC ?
16	Human breast cancer (5)	1.7	GP4 1/8	DES	minimal, stroma > cells.	?	?
17	Human breast cancer (6)	4.7	GP4 1/8	DES	Cells << stroma (++)	?	?
18	Cystosarcoma.	0	GP4 1/8	DES	+++	No	-
19	Human breast cancer (7)	0	GP4 1/8	DES	++	No	-
20	l. ovarian 2 <sup>0</sup> from human breast cancer.	7.35	GP3	omitted	+++	-	(+)
21	r. ovarian 2 <sup>0</sup> from human breast cancer.	1.71	GP4 1/8	DES	++	Partial	+
22	as above (2nd assay)	"	GP4 1/8	DES	none	-	-
23	as above (3rd. assay)	"	GP4 1/8	DES	none	-	-
24	as above (4th. assay)	"	GP4 1/8	DES	+	?	?
25	Benign mammary hyperplasia	0.6	GP4 1/8	DES	+	?	?





a. x 160

(Counterstain too pale for  
photographic reproduction.)

b.

Figure 29.

Frozen sections of immature rat vagino-cervical junction.  
(See Table 15, assay 1.)

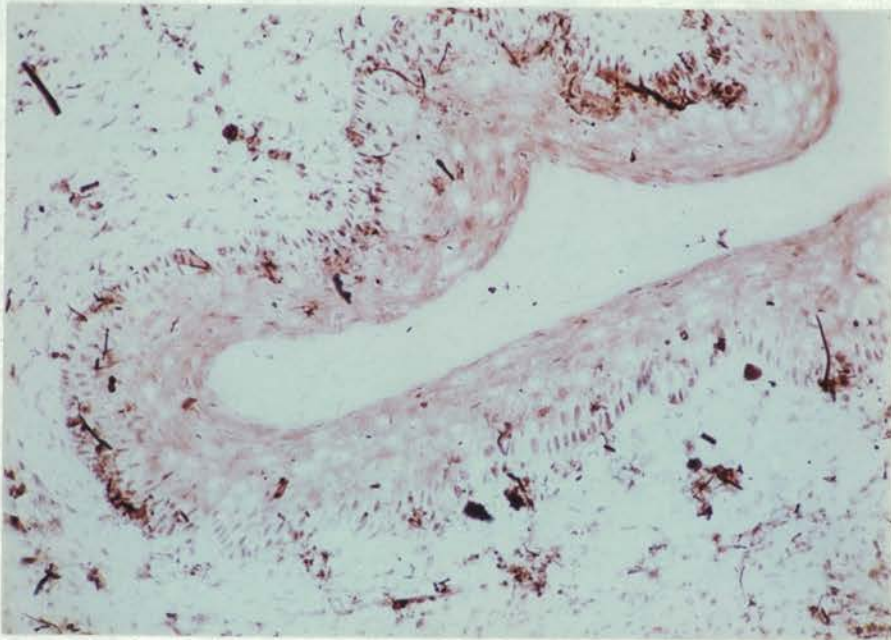
a) Toluidine blue stain to demonstrate tissue architecture.

b) DAB-H<sub>2</sub>O<sub>2</sub> stain to demonstrate endogenous peroxidase,  
Mayer's haematoxylin counterstain.





c.

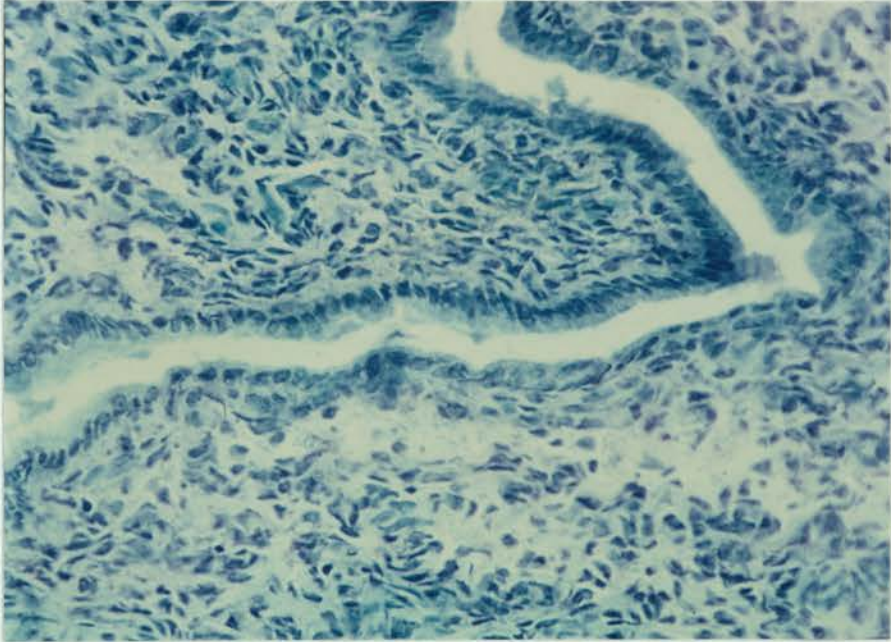


d.

Figure 29 (cont.)

- c) After incubation with oestradiol-BSA-peroxidase conjugate. Mayer's haematoxylin counterstain.
- d) After co-incubation with oestradiol-BSA-peroxidase conjugate and competitor (DES), Mayer's haematoxylin counterstain.





a. x 320

(Counterstain too pale for  
photographic reproduction.)

b.

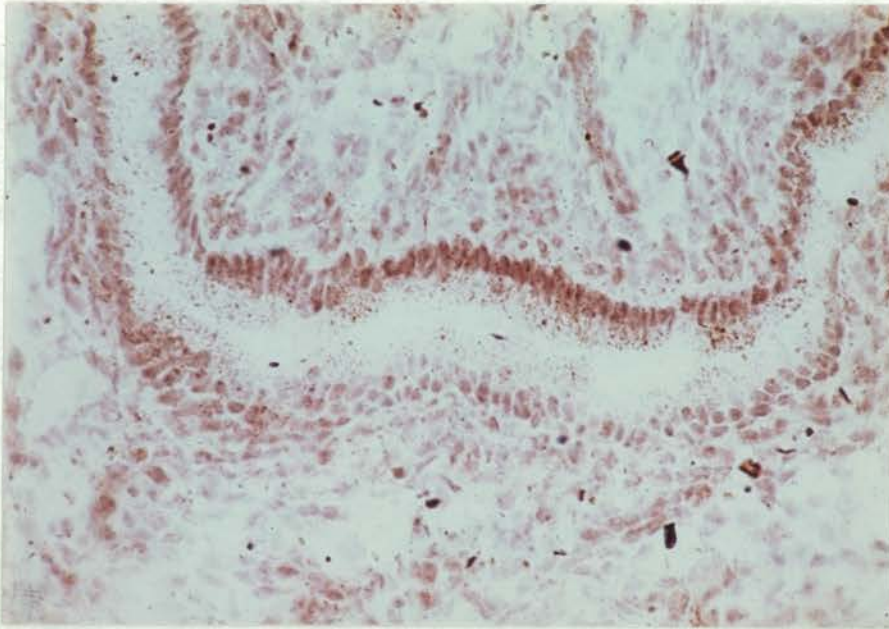
Figure 30.

Frozen sections of lactating rat uterus.

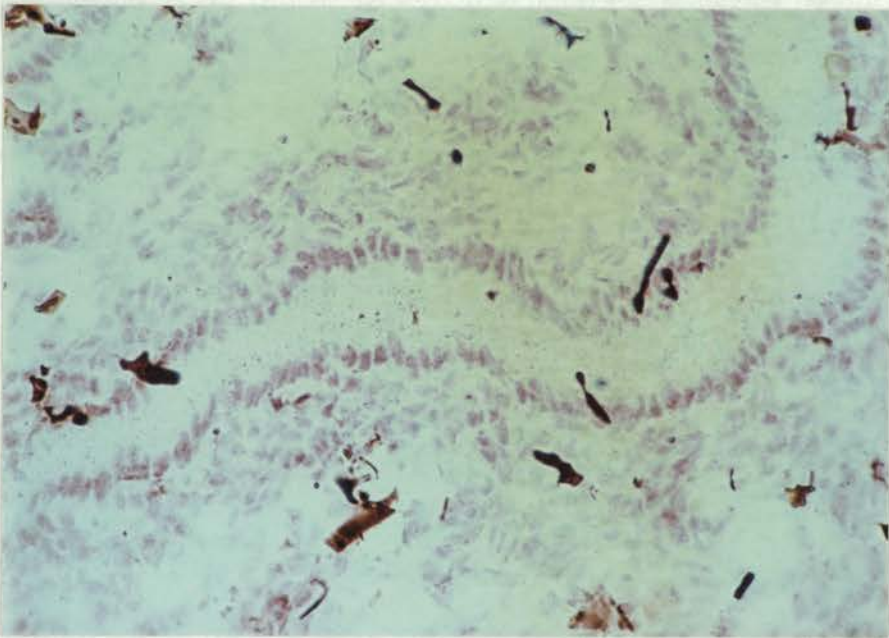
(See Table 15, assay 4).

- a) Toluidine blue stain to demonstrate tissue architecture.
- b) DAB-H<sub>2</sub>O<sub>2</sub> stain to demonstrate endogenous peroxidase,  
Mayer's haematoxylin counterstain.





c.



d.

Figure 30 (cont.)

- c) After incubation with oestradiol-BSA-peroxidase conjugate.  
Mayer's haematoxylin counterstain.
- d) After co-incubation with oestradiol-BSA-peroxidase  
conjugate and competitor (DES), Mayer's haematoxylin  
counterstain.



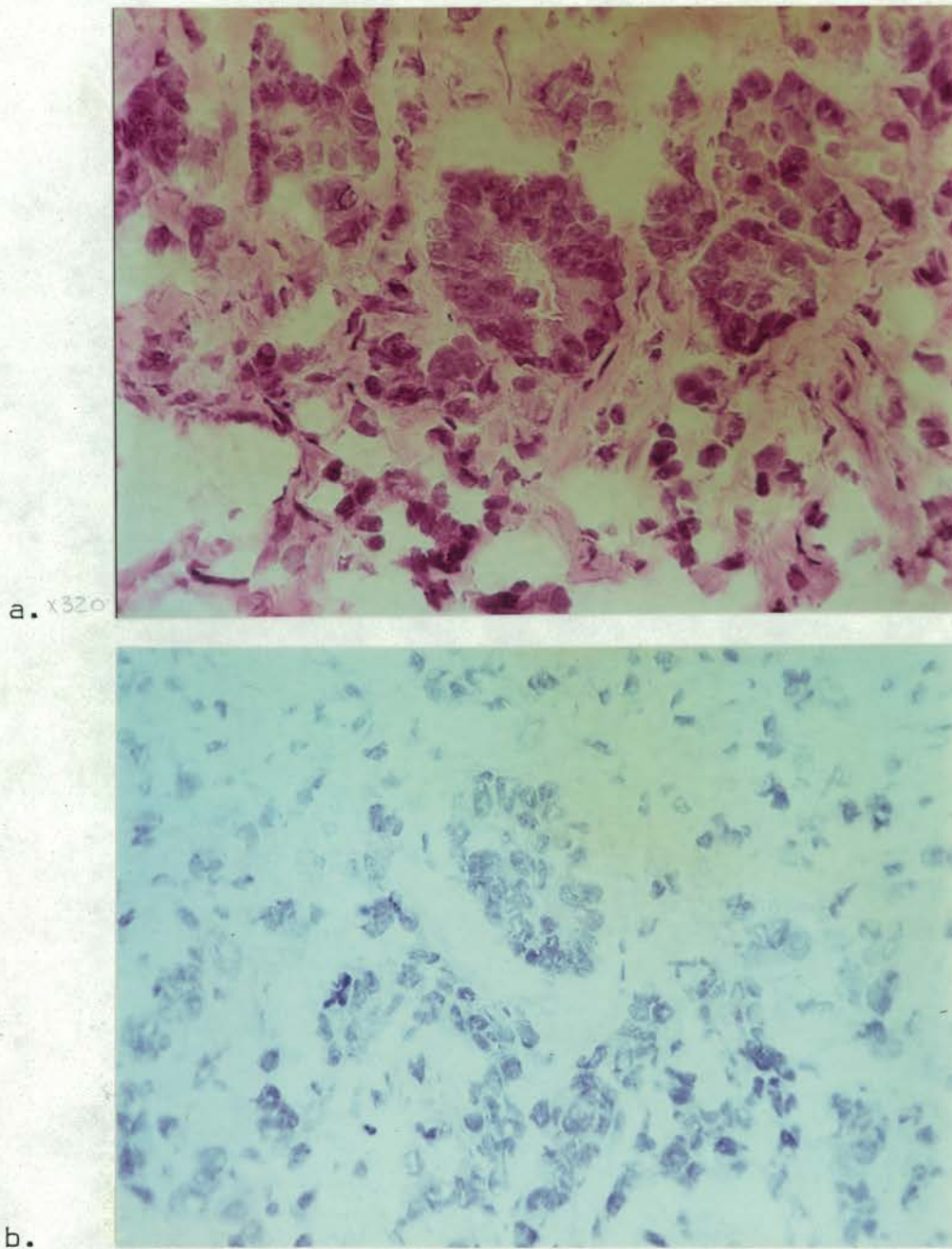


Figure 31.

Frozen sections of human breast cancer cells.

(See table 15, assay 12.)

- a) Haematoxylin & Eosin stain to demonstrate tissue architecture.
- b) DAB-H<sub>2</sub>O<sub>2</sub> stain to demonstrate endogenous peroxidase, Mayer's haematoxylin counterstain.

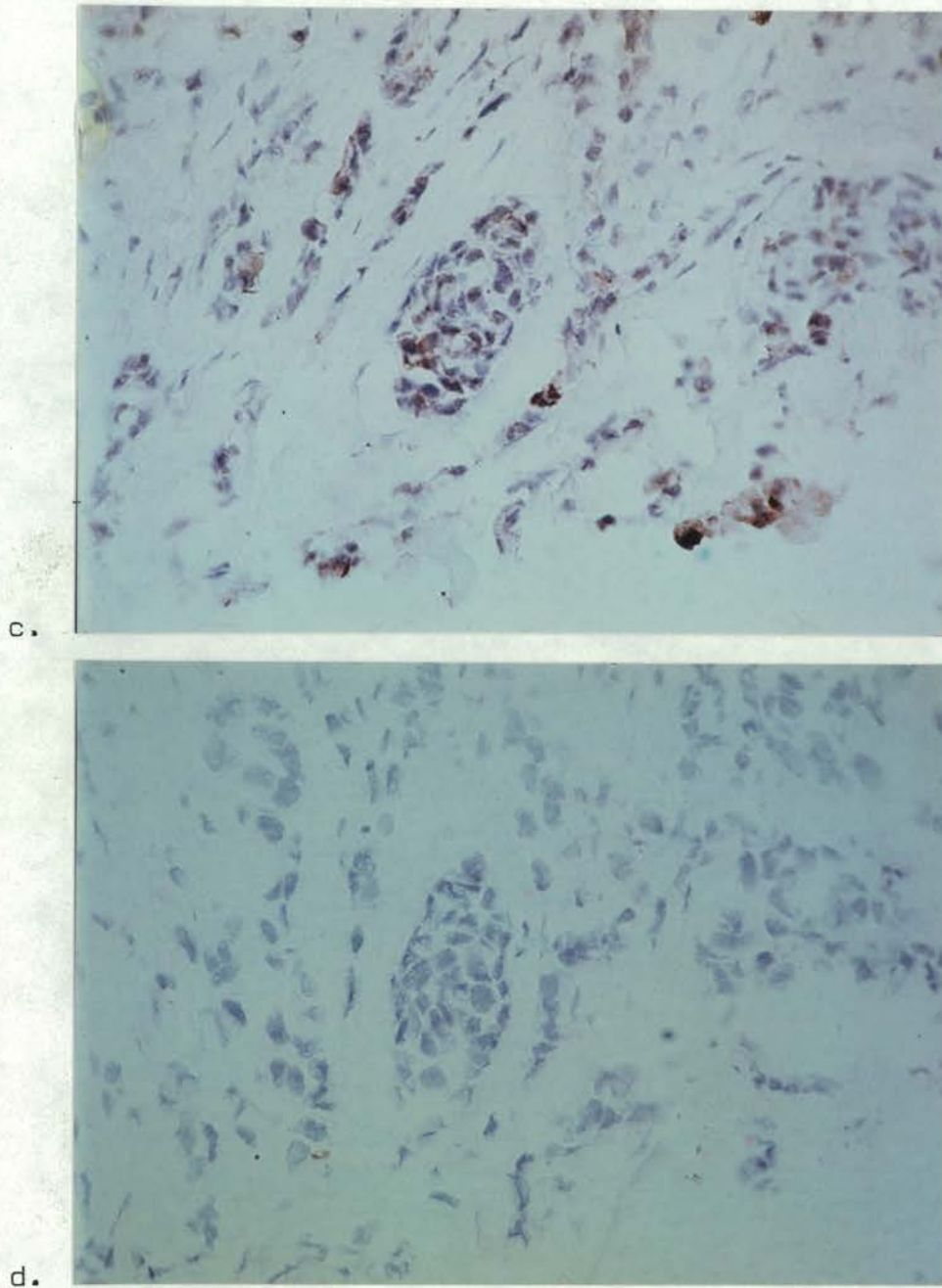
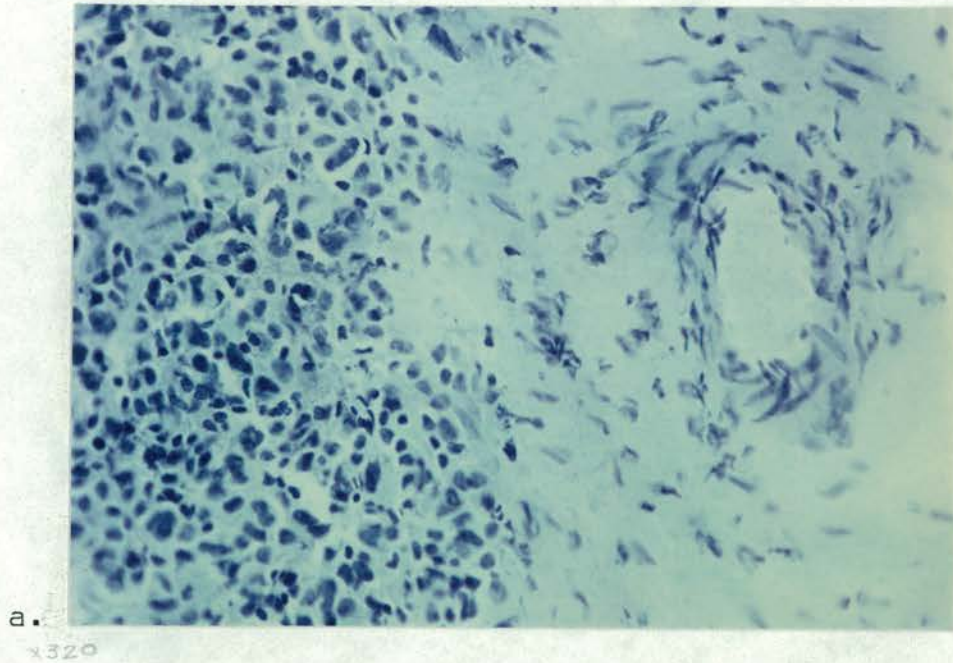


Figure 31 (cont.)

- c) After incubation with oestradiol-BSA-peroxidase conjugate. Mayer's haematoxylin counterstain.
- d) After co-incubation with oestradiol-BSA-peroxidase conjugate and competitor (Tamoxifen). Mayer's haematoxylin counterstain.





(Counterstain too pale for  
photographic reproduction.)

b.

Figure 32.

Frozen sections of ovarian metastasis from human breast cancer.

(See Table 15, assay 21).

- a) Toluidine blue stain to demonstrate tissue architecture.
- b) DAB- $H_2O_2$  stain to demonstrate endogenous peroxidase,  
Mayer's haematoxylin counterstain.

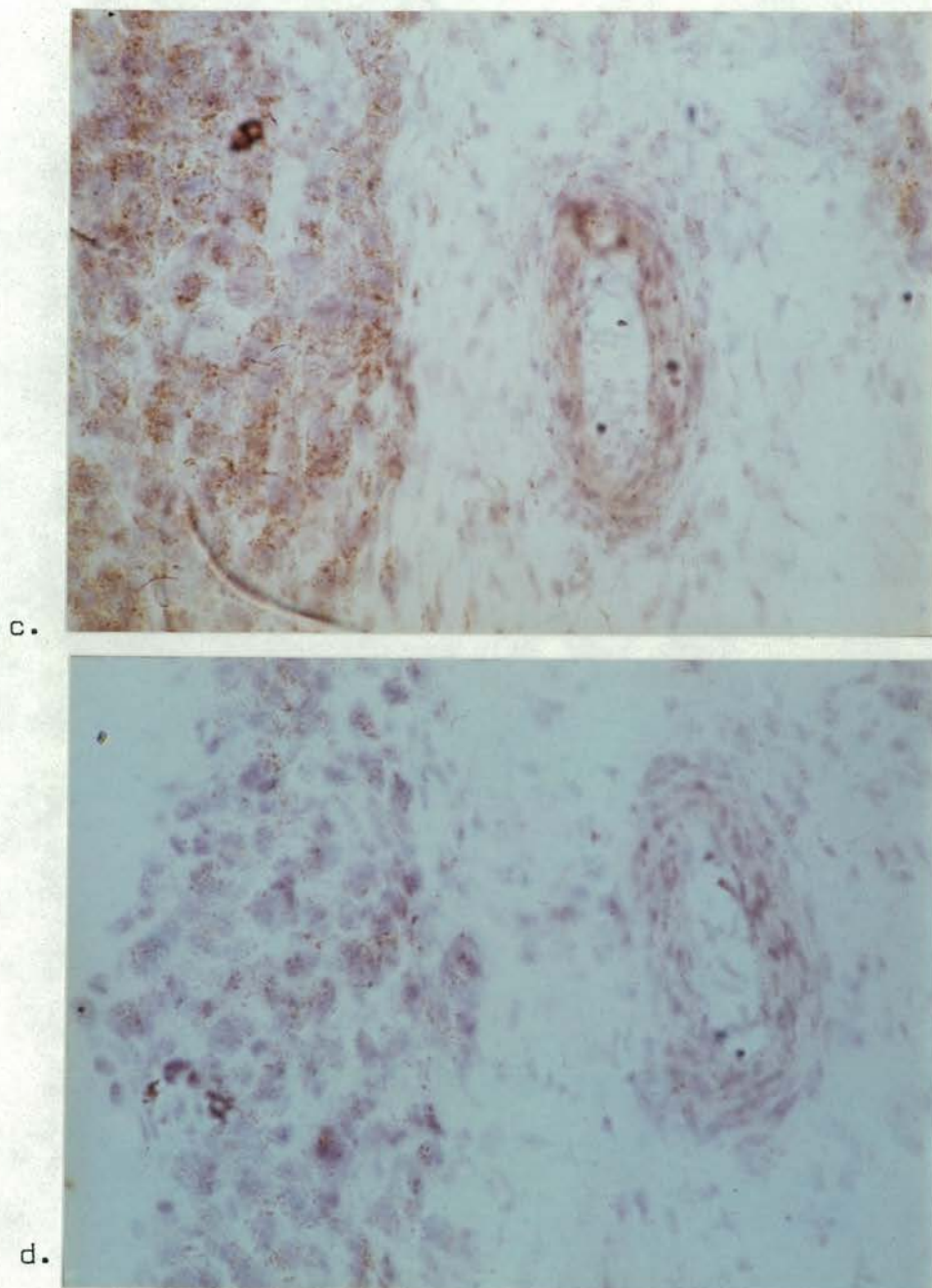


Figure 32 (cont.)

- c) After incubation with oestradiol-BSA-peroxidase conjugate, Mayer's haematoxylin counterstain.
- d) After co-incubation with oestradiol-BSA-peroxidase conjugate and competitor (DES), Mayer's haematoxylin counterstain.

PART IVDISCUSSION



# I. ENDOGENOUS PEROXIDASE AS A MARKER FOR HORMONE-DEPENDENCE

## A. Establishment Of Assay Techniques

The series of experiments described in "Results I" was undertaken to determine optimal substrate concentrations and assay conditions for a spectrophotometric peroxidase assay. The ranges of substrate concentrations tested were chosen by consideration of the levels used by other workers (Maehly and Chance, 1954; Himmelhoch et al., 1969; Putter, 1974; Lyttle and DeSombre, 1977) in similar assay systems. Graphs relating initial rate of reaction to substrate concentration were constructed for both guaiacol and hydrogen peroxide with a view to selecting substrate concentrations corresponding to maximum initial rates of reaction, i.e. concentrations adequate to "saturate" amounts of enzyme likely to be encountered in practice.

A value for Michaelis Constant ( $K_m$ ) with respect to guaiacol was readily determined and an assay concentration equivalent to  $9 \times K_m$  was selected. In the case of hydrogen peroxide, however, a distinct "plateau" region on the initial rate/concentration curve, indicating enzyme-saturation, was not obtained within the range of concentrations tested. Nevertheless, a value for  $K_m$  with respect to hydrogen peroxide was calculated from the straight-line (Lineweaver-Burke) plot of the assay data. The chosen assay concentration proved to be approximately equal to  $K_m$ , and although far from ideal in theory, was similar to that used by Lyttle and DeSombre (1977) in their assay. It is, perhaps, not surprising that this difficulty in the selection of peroxide concentration arose as Putter (1974) has stated that the kinetics of the guaiacol reaction are such that a true value of  $K_m$  with respect to hydrogen peroxide probably cannot be determined.

Further preliminary experiments served to ensure that the optimal method of estimating "initial" rate of reaction was chosen, and to demonstrate that the assay

"precision" (intra-assay variation) was good (approximately 7%). In addition, the assay was validated by demonstration of linear relationships between increase in absorbance measured under the routine conditions and known concentrations of both horseradish and uterine peroxidases. Finally, the stability of peroxidase activity in liquid nitrogen storage was established.

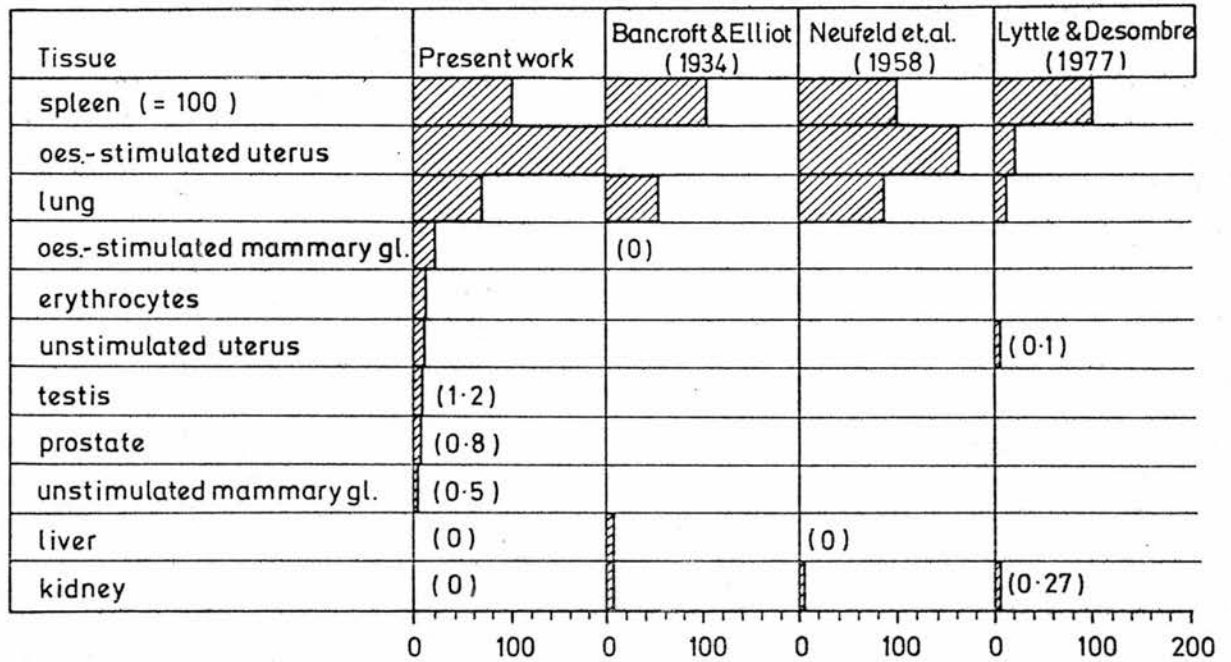
## B. Peroxidase Activity In Normal Rat Tissues

The study described in "Results II" involved the measurement of peroxidase activity in a range of normal rat tissues; namely liver, kidney, prostate, testis, erythrocytes, spleen, mammary gland and uterus during various reproductive states. Peroxidase activity was detectable in all the tissues examined except liver and kidney, the highest values being found in uterus. Several groups of other workers have presented data on peroxidase activity in normal rat tissues in the past. In 1934, Bancroft and Elliot used a rather crude assay technique, without the benefit of calcium chloride extraction and using pyrogallol as hydrogen donor. In contrast with the present study, they detected peroxidase, in small amounts, in liver and kidney, but found it to be absent from mammary gland. They found the highest levels in spleen and lung, but did not examine the uterus.

In a later study, Neufeld et al. (1958) examined a larger range of tissues. In agreement with the present work, they could not detect peroxidase in liver, found high levels in the uterus and intermediate levels in spleen and lung. However, they were able to detect a low activity in kidney and found the highest levels in small intestine, which was not examined in the present study. Lyttle and DeSombre (1977) have also assayed a range of normal rat tissues. They found the greatest activity in spleen, high levels in oestrogen-stimulated uterus and also in lung, a low level in kidney, and, as in the present study, found no activity in liver.

In general, therefore, the results of this study are in agreement with those of other groups: endogenous peroxidase is detectable in a wide range of normal tissues of the rat, both classical oestrogen-target tissues such as uterus, and non-target tissues such as spleen. In the target tissues, uterus and mammary gland, peroxidase levels are higher when the tissue is under oestrogen-stimulation (during oestrus or

after administration of exogenous hormone) than in the unstimulated tissue (during lactation or after castration). This finding indicates that endogenous peroxidase does seem to serve as a marker for oestrogen-target cell interaction. The relative levels of peroxidase found in the various tissues in this study are similar to those found by the other groups of investigators. The comparison is summarised in Figure 33.



**Figure 33 .**

Levels of peroxidase detected in a range of normal rat tissues by various investigators. Because of the differing assay techniques of the various groups, results have been expressed as a % of the result obtained for spleen by each group. Where no value is shown, that tissue was not examined by that particular group of authors.

C. The Relationships Between Levels Of Peroxidase,  
E/R And Pg/R In Normal Rat Tissues.

In the study described above, E/R and Pg/R levels were measured, in addition to peroxidase activity, in a range of normal rat tissues. Like peroxidase, E/R was found to be very widely distributed, being detectable in all the tissues examined except erythrocytes. Stumpf and Sar (1976) have also estimated the E/R content of normal rat tissues, using an autoradiographic technique. In addition to the classical oestrogen-target tissues (uterus, vagina, oviduct, mammary gland, anterior pituitary, hypothalamus) they were able to detect oestrogen-target cells in testis, prostate, kidney and liver, all of which were examined, and found to be E/R-positive, in the present study. Stumpf and Sar examined an exhaustive range of tissues and, in addition to those mentioned above, reported oestrogen-binding in thymus, adrenal, many parts of the male reproductive tract, heart, diaphragm, salivary glands, arterial walls and central nervous system.

In the present work, Pg/R was found to be much less widely distributed, being present only in the classical target-tissue, uterus, in lung and in kidney. Stumpf and Sar (1976) studied progestogen-binding by means of autoradiography and reported the finding of target cells in female reproductive organs and in the pituitary but not in central nervous tissue nor, to their satisfaction, in liver.

Thus, in general, peroxidase, E/R and, to a lesser extent, Pg/R are widely distributed in normal rat tissues, the highest levels of all three proteins being found in the classical oestrogen-target tissue, the uterus. In target tissues (uterus and mammary gland), the levels of all three proteins vary depending on the reproductive status of the host animal. Detectable E/R levels are highest when circulating oestrogen is low (e.g. during lactation), but relatively low during oestrus when high endogenous oestrogen

levels "block" E/R from binding to radiolabelled-oestrogen. In contrast, both Pg/R and peroxidase levels are highest during oestrus and relatively low during lactation, lending support to the hypothesis that synthesis of both these proteins is dependent on oestrogen - E/R interaction.



D. Peroxidase, E/R And Pg/R As Markers For Hormone-Dependence In Rat Mammary Tumour Models.

Following the implication from the study of healthy rat tissues, that peroxidase does seem to serve as a marker for oestrogen-dependence, this property was further investigated in a series of rat mammary tumours which serve as models for hormone-dependent and -independent growth (Results III). The tumour models have been validated in a previous study from this department (Scott et al., 1979). These workers showed that all of 12 DMBA-induced rat mammary tumours regressed after oophorectomy, whereas none of 12 TG3 tumours and none of 6 TG5 tumours did so. Hawkins et al. (1978) have presented typical growth curves for each of the 3 types of tumour. In view of these data, DMBA-induced tumours were accepted as models for hormone-dependent growth and transplantable tumours of TG3 and TG5 lines were accepted as models for hormone-independent growth for the purposes of the present study.

In these tumours, E/R content proved to be a "perfect" marker for hormone-dependence, there being no "overlap" whatsoever in the ranges of levels encountered in a group of 12 DMBA-induced tumours and in a group of 12 transplantable tumours. This difference is more clear-cut than that found in a previous report from this department (Hawkins et al., 1978) of 10 DMBA-induced and 12 transplantable tumours. In that report, a significant difference ( $p < 0.01$ ) was found between the E/R levels in the two types of tumour but there was considerable overlap between the groups, with the levels found in DMBA-induced tumours being much lower than those observed in the present study. This discrepancy may be explained, at least in part, by the fact that the host animals were not exsanguinated prior to removal of the tumour specimens and high blood content can interfere with E/R assay techniques (Hawkins et al. 1978). Certainly, in a previous study (Hawkins et al., 1977), where DMBA-induced tumours alone were examined, the range of levels of E/R



found were closer to those found by the present author. In a further study from this department (Scott et al., 1979) a significant difference ( $p < 0.01$ ) was found between the two types of tumours but, again, the ranges were not as distinct as in the present study.

Pg/R also emerged as an excellent marker for hormone-dependence. For this protein, the ranges of levels found in the two groups were, again, quite distinct with the exception of a single DMBA-induced tumour in which no Pg/R was detectable. It might be postulated that this tumour may have belonged to the hormone-independent minority of DMBA-induced tumours.

In the case of peroxidase, there was a highly significant difference ( $p < 0.001$ ) between the levels found in the two types of tumour, confirming that peroxidase does serve as a marker for hormone-dependence. However, there was considerable overlap between the ranges of values found in the two groups. Under the conditions of this study, therefore, endogenous peroxidase would seem to be inferior to both E/R and Pg/R as a marker for hormone-dependence. Lyttle and DeSombre (1979) used a biochemical assay very similar to that of the present author to measure peroxidase levels in mouse mammary tumour models for hormone-dependent and -independent growth. In their study, there was no overlap between the ranges of peroxidase levels encountered in the two groups of tumours indicating that, under some circumstances, peroxidase serves as an excellent marker for hormone-dependence.

E. Peroxidase As A Marker For Hormone - Dependence  
In Human Mammary Cancers.

The finding of a relationship between peroxidase levels and hormone-dependence in rat mammary tumour models prompted the evaluation of peroxidase as a marker for hormone-dependence in a series of human cancers (Results IV). Peroxidase activity was detectable in 11 of 17 (65%) tumours examined. This incidence of "peroxidase-positivity" is similar to those quoted by other investigators: 77% of 39 tumours (Lyttle and DeSombre, 1977), 62% of 52 tumours (Duffy and Duffy, 1978) and 68% of 130 tumours (Collings and Savage, 1979).

The incidence of "E/R-positivity" found in the present series was 65% and, again, this figure is similar to those generally reported by others (Hawkins et al., 1980). There was, however, no relationship between tumour peroxidase content and either E/R status or absolute level of E/R. In particular, of 6 tumours designated E/R-negative, 4 were peroxidase-positive. As the predictive value of negative E/R status is well established, this finding suggests that peroxidase is unlikely to be a useful marker for hormone-dependence in human cancers. Duffy and Duffy (1978), in a series of 52 cancers, did find a relationship between peroxidase content and E/R status: 78% of E/R-positive tumours contained peroxidase compared with only 20% of E/R-negative cases. However, Collings and Savage (1979), in their much larger series of 130 tumours, were unable to demonstrate any such relationship. Their finding that 71% of E/R-negative tumours contained significant peroxidase activity more closely resembles the present work and must be regarded as discouraging.

Data concerning response to endocrine therapies was available for only 3 of the 17 cases in the present study and can only be regarded as anecdotal. However, the finding of the highest peroxidase level in an E/R-negative, endocrine-unresponsive tumour, again suggests that peroxidase assays are unlikely to prove clinically useful.

F. The Histochemical Assessment Of Peroxidase Activity  
In Rat Mammary Tumour Models.

The studies discussed above utilised a biochemical assay for the measurement of peroxidase activity in tissue extracts. Some investigations into the properties of oestrogen-induced peroxidase have used an alternative, histochemical technique; such studies include work on the uterus by Brockelmann and Fawcett (1968) and by Anderson et al., (1975) and also work on rat mammary tumours by Anderson et al. (1975) and by DeSombre et al. (1975). In these studies, stained sections were examined at the electron microscope level in order to determine the subcellular distribution of the peroxidase. As a result, DeSombre et al. (1975) suggested that identification of peroxidase at light microscope level might be an indication of hormone-dependence in mammary tumours.

In the present work, this possibility was evaluated by comparing the peroxidase contents of rat mammary tumours after assessment by both biochemical and histochemical methods (Results V). Peroxidase levels determined biochemically were significantly higher in the hormone-dependent than in the hormone-independent tumours. This finding is in agreement with the results of the previous study (Results III). It was surprising, however, to find an inverse relationship between peroxidase activity as assessed histochemically and hormone-dependence! Moreover, those tumours designated histochemically positive for peroxidase had significantly lower biochemical values than those designated histochemically negative.

Both the guaiacol and diaminobenzidine reactions are well established techniques for the demonstration of peroxidase activity, and this great discrepancy between the results of the two methods in the rat mammary tumour models is difficult to explain. It is possible that differences in the preparation of tissues for the two techniques result in

differing levels of enzymes such as oxidases and catalases which may interfere with peroxidase assay systems. Irrespective of the cause, however, this paradoxical finding indicates that extrapolation cannot be made from a correlation between biochemical estimates of peroxidase and hormone-dependence to a clinically useful test based on histochemical examination of frozen sections.

G. Conclusions

The Present Status Of Peroxidase As A Marker  
For Hormone-Dependence

The studies presented in this thesis allow the following conclusions to be drawn concerning the possible role of peroxidase as a marker for hormone-dependence:

1. Peroxidase is widely distributed in normal rat tissues, both classical oestrogen-target tissues such as uterus and non-target tissues such as spleen.
2. In common with the established "marker-proteins", E/R and Pg/R, high levels of peroxidase are found in the uterus. In addition, the finding here and in the work of others, that uterine peroxidase, in parallel with Pg/R, is highest in the presence of maximal "oestrogen-E/R interaction" lends support to the hypothesis that peroxidase synthesis is induced by an oestrogen-mediated effect on the genetic material of the cell nucleus.
3. Peroxidase content in rat mammary tumour models is significantly related to hormone-dependence. However, peroxidase is inferior, as a marker, to both E/R and Pg/R.
4. In contrast, the currently available evidence indicates that peroxidase does not serve as a marker for hormone-dependence in human mammary cancers.
5. In rat mammary tumour models, there is no direct relationship between biochemical and histochemical estimates of peroxidase activity. This suggests that the two assay systems reflect different properties and that staining of frozen sections for peroxidase is unlikely to prove useful in the prediction of hormone-dependence.

In summary, much evidence exists to indicate that peroxidase-synthesis in oestrogen-target tissues is stimulated by an oestrogen-mediated effect on the genetic material of the cell nucleus. Unfortunately, assay of peroxidase activity would seem to be inferior, in practice, to assays of cytoplasmic E/R and of Pg/R, for predicting hormone-dependence. In addition, biochemical and histochemical estimates of peroxidase activity differ markedly. These observations make it seem unlikely that peroxidase will prove clinically useful in assessing endocrine-responsiveness in mammary cancers.

## II. A HISTOCHEMICAL APPROACH TO THE IDENTIFICATION OF OESTROGEN RECEPTORS

In the absence of a currently available, specific antibody to the E/R protein, two approaches to the identification of E/R in tissue sections are available. Indirect immunohistological methods utilising an antibody raised against oestradiol have been investigated by many groups, notably Nenci et al. (1976), Malan & Janss (1978), Mercer et al. (1978<sup>ab</sup> & 1979<sup>ab</sup>), Pertschuk et al. (1978) and Walker et al. (1980). A second approach involves the use of histochemical staining techniques. Those groups who have investigated both immunohistological and histochemical methods (Pertschuk et al., 1979; Walker et al., 1980) both report that the latter give superior results. Because of the views expressed by these investigators, histochemical rather than immunohistological techniques were selected for evaluation in the present work.

### A. Quantitation Of Losses Of E/R Activity During Histochemical Processing.

In the series of experiments described in "Results VI" an attempt was made to quantitate the possible losses of E/R activity occurring during various processes which are components of histochemical techniques. It would appear that even the simple manoeuvre of dividing a portion of tissue into frozen sections may result in the loss of over 50% of measurable E/R activity. The cutting of frozen sections of thickness 4  $\mu\text{m}$  (as recommended by Pertschuk et al., 1979), or even 14  $\mu\text{m}$  (as recommended by Lee, 1979), inevitably results in the transection of many cells. It is possible that this cellular disruption permits a portion of the soluble cytoplasm, of which E/R is a component, to escape onto the cryostat knife. A second possible explanation for the loss of E/R activity observed may be that section-cutting



results in local heat production at the section surface with consequent damage to the thermolabile E/R protein.

Following the cutting and mounting of the frozen-sections, the next step in most histochemical techniques is some form of fixation to ensure adhesion of the sections to the glass and to achieve "immobilisation" of proteins within the section. In view of the possible deleterious effects of conventional fixatives on E/R activity, many investigators have dispensed with fixation at this stage and have relied, simply, on a period of "air-drying" (Dandliker et al., 1978; Lee, 1978; Pertschuk et al., 1979). Lee admits that this failure to effectively "immobilise" the E/R protein may result in a loss of activity:

"Conceivably, an undetermined amount of receptor protein that is not firmly bound to the sub-cellular structures might become dissociated from the tissue sections. This loss, if any, is considered inevitable due to limitation of the methodology."

The experiment described on p. 110 was designed in an attempt to **quantitate just such** losses from unfixed frozen sections into an aqueous medium analogous to the steroid-containing incubation medium of histochemical techniques. Under the conditions of this experiment, 66% of E/R appeared to "leach-out" from 14  $\mu$ m sections, and 75% from 4  $\mu$ m sections, into the aqueous medium. Losses of this magnitude are probably well in excess of those which would occur in a genuine histochemical technique; in the model system used the sections were unmounted and were therefore exposed to the medium on both surfaces, and also the process of separating sections from medium was somewhat less gentle than would be achieved under histochemical conditions. Nevertheless, it would appear that very significant losses of E/R activity might occur when unfixed frozen sections are exposed to aqueous processing media.



Walker et al. (1980) adopted a more conventional approach to immobilisation of proteins, fixing their frozen sections by immersion in acetone for 4 min. prior to incubation with peroxidase-labelled oestradiol. The present author was concerned that such treatment might denature the E/R protein, resulting in loss of activity. The experiments described on p. 112 were undertaken in an attempt to quantitate these losses. Initially, cytosols prepared from acetone-fixed, rat uterine fragments were assayed for E/R activity by routine DCC assay and were found to contain minimal E/R. It was realised, however, that if acetone-fixation had successfully immobilised the E/R protein, E/R activity would not be detectable in the cytosol but would remain in the homogenisation pellet. Subsequently, therefore, the homogenisation pellets from acetone-fixed and from unfixed uterine fragments were assayed for E/R by a "solid-phase" technique. In the case of unfixed tissue, no E/R was demonstrable in the pellet preparation, indicating that, in the unfixed state, all the E/R detectable by the techniques used is localised in the soluble cytoplasm. In the case of acetone-fixed tissue, a small amount of E/R activity was detectable in the pellet preparation. This finding suggests that acetone-fixation may be a valid step in histochemical E/R assays; however, both the concentration ( $P_0$ ) and the dissociation constant ( $K_d$ ) were markedly impaired in comparison with those of E/R in the cytosol of unfixed tissue. The loss of E/R activity attributable to acetone-fixation appeared to be about 50%, similar to the magnitude of loss caused by "leaching - out" of E/R in the case of unfixed tissues.

Lee (1978) similarly reported that exposure of tissues to acetone (at  $-20^{\circ}\text{C}$ ) results in almost complete abolition of cellular, oestrogen binding capacity. Lee also investigated 3.7% buffered formaldehyde and 1% glutaraldehyde as possible fixatives, but felt that these substances also resulted in loss of E/R activity and that

air-drying was the most practical approach to E/R immobilisation. Dandliker et al. (1978) investigated the effects of 4% formalin (1% formaldehyde) on E/R activity in a quantitative manner. They estimated that E/R activity in formalin-fixed tissues was only 20 - 40% of that detectable in equivalent, untreated tissues. These investigators also concluded that air-drying gave the best results in practice.

Lee (1978) did not expose frozen sections to fixative agents at any stage of his histochemical technique; Pertschuk (1979), however, employed "post-fixation" in ethanol/acetone after the incubation of sections with fluorescein-conjugated oestradiol. The present author was concerned that exposure to such a fixative at this stage might result in breakage of the bonds between E/R and the labelled oestradiol. The experiment described on p.116 demonstrated that such a breakage does, in fact, occur when tritiated oestradiol is used as a tracer. It might be argued that this breakage of the oestradiol-E/R bond is unimportant when an albumin-containing tracer, as in Pertschuk's technique, is used because the fixation would serve to immobilise the protein conjugate at the site of E/R interaction. However, such a mechanism would also result in the immobilisation of any non-specifically deposited conjugate and would, presumably, prevent it from being removed by subsequent washing procedures.

In summary, this preliminary series of experiments has indicated that significant losses of E/R activity might be expected to occur during the actual cutting of frozen sections, during exposure of unfixed tissues to aqueous media and during exposure to fixatives, either before or after the incubation of sections with oestradiol linked to a visible tracer. These possible losses of activity during histochemical methods for E/R determination are summarised in Table 16 .

Table 16.

Summary of the sites and magnitude of losses of E/R activity which may occur when tissues are exposed to histochemical processing.

PROCESS	AUTHOR	% LOSS
Section cutting	Present Work	50
Exposure of unfixed tissue to aqueous media.	"	66-75
Fixation in acetone	"	50
"	Lee (1978)	"almost total"
Fixation in glut-araldehyde.	"	"
Fixation in form-aldehyde.	"	"
"	Dandliker et al (1978)	60-80
"post-fixation" in etanol/acetone	Present work	"almost total"

## B. Possible Approaches To Minimising Losses Of E/R Activity

In view of the finding that losses of E/R activity of up to 50% might accrue from exposure to conventional fixatives or from exposure of unfixed tissues to aqueous media, two alternative approaches to protein-immobilisation were investigated. Both ammonium sulphate and protamine sulphate are reported to precipitate E/R into a solid-phase without loss of activity (Dandliker et al., 1978; Steggles and King, 1970). Preliminary studies employing radioactive oestrogen as tracer were undertaken to establish whether or not either of these compounds might prove useful for the immobilisation of E/R in a histochemical situation.

In the experiment described on p.119 ammonium sulphate was found to be very effective in preventing the "leaching-out" of E/R from unfixed frozen sections. In this study, 82% of E/R activity leached out into an aqueous medium in the absence of ammonium sulphate, but this loss was reduced to 10% by the presence of 50% saturated ammonium sulphate. Unfortunately, when such treatment was applied to the assay of E/R in glass-mounted frozen-sections (p.131 ), a situation more closely resembling an actual histochemical technique, results proved less satisfactory. The addition of ammonium sulphate to processing media in this system resulted in very high "non-specific" binding which was not blocked by coincubation with unlabelled steroid.

The preliminary work with protamine sulphate (p.123 ) served to confirm the finding of Lippman and Huff (1977) that E/R can be precipitated by protamine without loss of activity. In the present study the concentration ( $P_0$ ) of E/R was unimpaired by precipitation but, unlike Lippman and Huff, the present author found the strength of binding (as reflected by  $K_d$ ) to be diminished. When protamine-treatment was used in the glass-mounted system (p.133 ) some E/R activity was demonstrable; high non-specific binding such as had occurred with ammonium sulphate did not

arise. However, the differential labelling between sections incubated with [ $^3\text{H}$ ]  $\text{E}_2$  alone and those coincubated with  $^0\text{E}_2$  achieved after protamine-treatment was inferior to that achieved after acetone-treatment using the same assay system. Using protamine, less than 50% of total [ $^3\text{H}$ ]  $\text{E}_2$  uptake could be attributed to "specific" labelling, compared with up to 86% using acetone-treatment.

In summary, most investigators in the field of E/R histochemistry employ one of two approaches to the problem of E/R-immobilisation without inactivation. Either a conventional fixative, such as acetone, is used or fixation is avoided and sections are simply air-dried. The author has investigated these two approaches and also two novel approaches (ammonium sulphate and protamine sulphate precipitation) in a model for a histochemical technique using the well-established tracer, [ $^3\text{H}$ ] oestradiol. Under these conditions, acetone-fixation produced more clear-cut and reproducible differential labelling than the other approaches. However, acetone fixation results in the loss of much activity before the receptor can finally be visualised and, hence, assays incorporating such a step cannot exhibit optimal sensitivity.

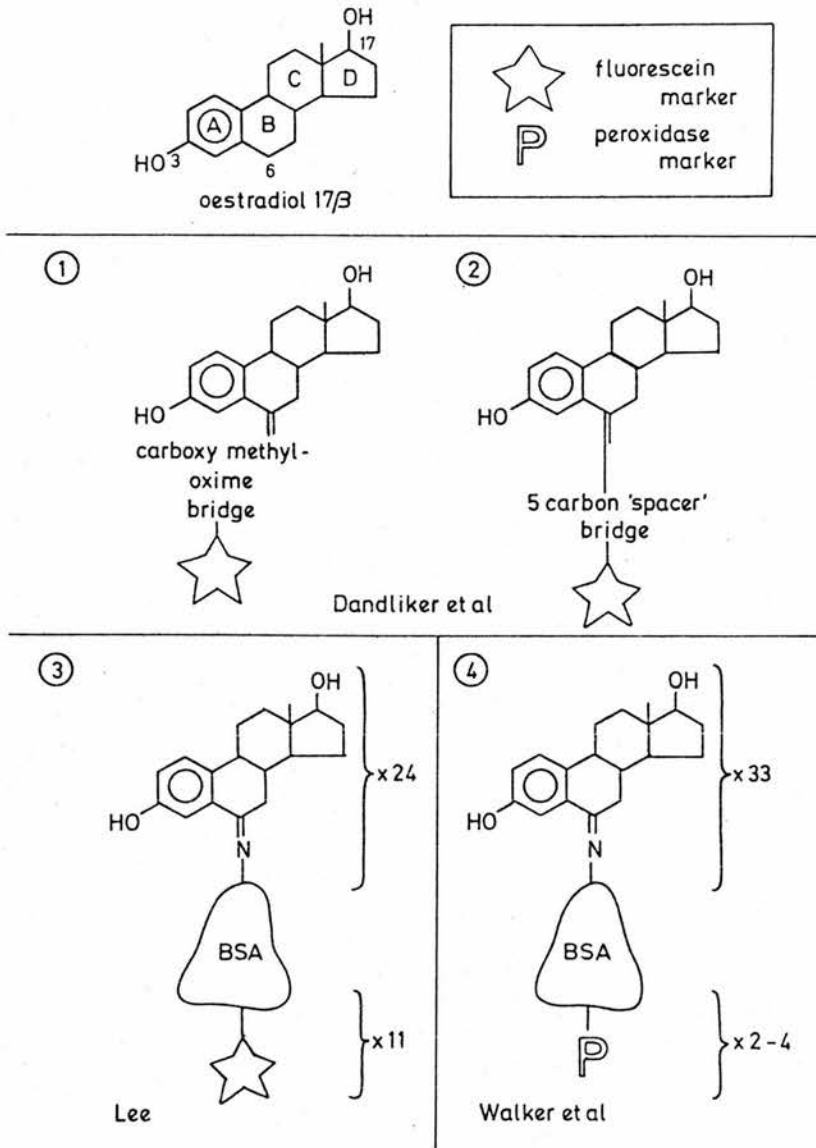
### C. Selection Of Oestradiol Conjugates.

A range of conjugates of oestradiol with a visible tracer have been advocated by investigators in the field of E/R histochemistry. The conjugates described can be divided into two main groups: those where oestradiol is linked to the tracer via the C<sub>6</sub> position and those linked via the C<sub>17</sub> position. Important examples of these two groups of conjugates are illustrated in Figures 34 and 35 .

The nature of the bond between oestradiol and its receptor is not yet fully elucidated, but Ellis and Ringold (1971) indicate that the hydroxyl groups at the C<sub>3</sub> and C<sub>17</sub> positions on the oestradiol molecule are involved in binding. If, in fact, this is the case then conjugation at the C<sub>6</sub> position, which leaves the hydroxyl group at C<sub>17</sub> intact, would be expected to produce a conjugate with superior binding properties to one utilising the C<sub>17</sub> position. A further theoretical point in favour of the C<sub>6</sub> position as the site of conjugation is that linkage to albumin at this position retains the antigenic specificity of the oestradiol molecule (Lindner et al., 1972) and might, therefore, be expected to retain functional specificity also. Figure 36 illustrates work from this department confirming the antigenic similarity between oestradiol 17 $\beta$  and C<sub>6</sub>-linked derivatives. Lee (1978) quoted this antigenic similarity as his rationale for the selection of a "C<sub>6</sub>" conjugate.

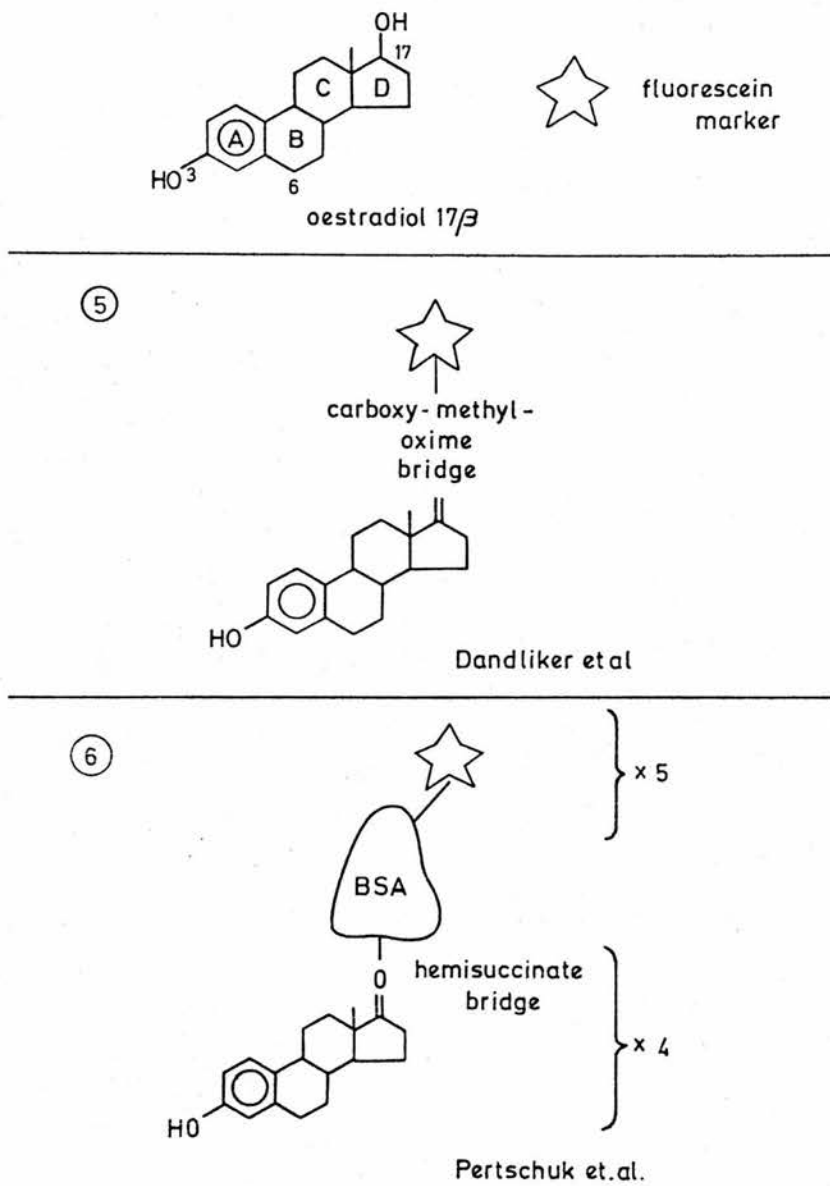
Dandliker et al. (1978) synthesised a range of oestradiol-fluorescein conjugates and measured their binding affinities to E/R in comparison with oestradiol 17 $\beta$ . Their original conjugate, known as 6-FE (compound 1, Fig. 34 ) was found to have a relative binding affinity of less than 0.01. They experimented with the insertion of a "5 carbon spacer bridge" in the 6-FE molecule (compound 2, Fig. 34 ) but still found the relative binding affinity to be less than 0.01. Despite the theoretical objections, Dandliker and co-workers then synthesised the compound 17-FE





**Figure 34. .**

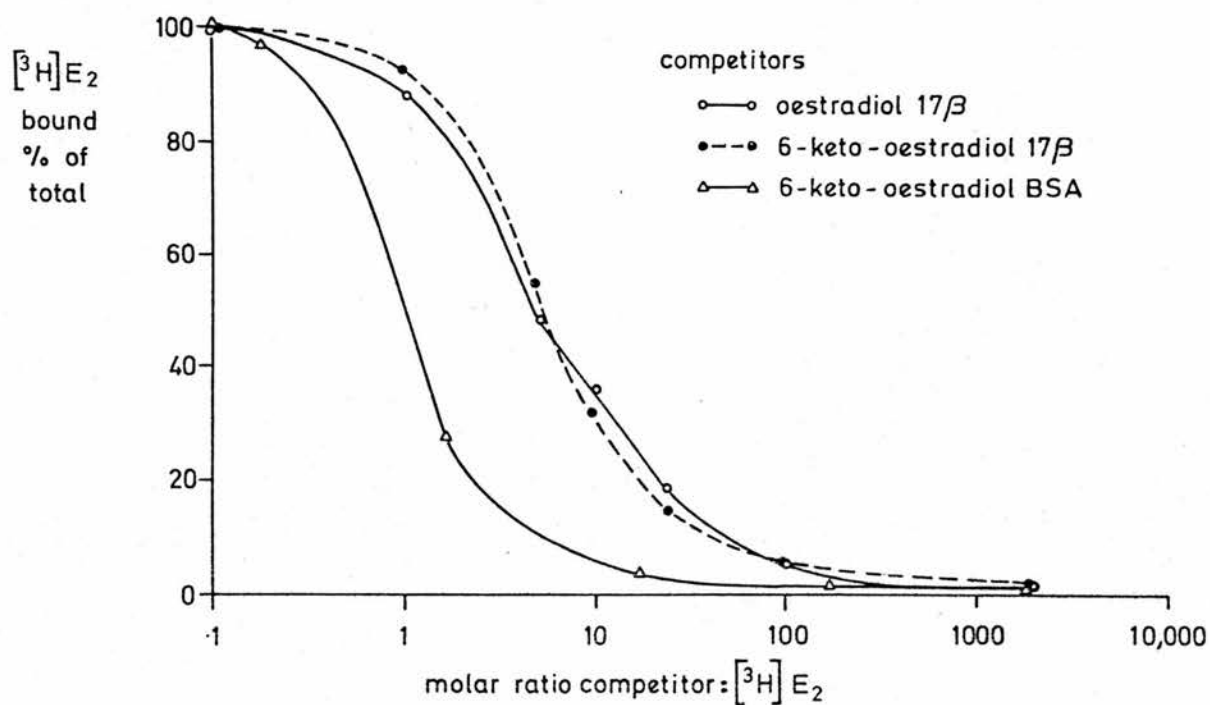
Oestradiol conjugates involving conjugation at the C<sub>6</sub> position which have been described as useful for the histochemical localisation of E/R. The parent molecule, oestradiol-17B, is also shown.



**Figure 35 .**

Oestradiol conjugates involving conjugation at the C<sub>17</sub> position which have been described as useful for the histochemical localisation of E/R. The parent molecule, oestradiol-17 $\beta$ , is also shown.





**Figure 36 .**

Relative affinities of Oestradiol 17B, 6-keto-oestradiol 17B and 6-keto-oestradiol 6-CMO-BSA for binding to an antibody generated against 6-keto-17B oestradiol-6-CMO-BSA.

Varying masses of each compound were allowed to compete with a fixed mass of (<sup>3</sup>H)E<sub>2</sub> ( 0.037 pmoles.) during binding, overnight, to rabbit antiserum at a final dilution of 1/98,400.

(This assay was kindly performed by Miss K Sangster.)

(compound 5, Fig. 35) and obtained a relative affinity of 0.03 which is, at least marginally, more acceptable. Very recently, Barrows et al. (1980) have published their experiences with a similar oestradiol-fluorescein conjugate utilising the C<sub>17</sub> position for conjugation. They reported a relative binding affinity approaching 0.1.

The site on the oestradiol molecule utilised for conjugation is only one point of difference between the various conjugates which have been used for E/R histochemistry. A second variable is that some investigators have employed simple oestradiol-fluorescein conjugates, such as the 6-FE and 17-FE of Dandliker et al. (1978), whereas others have linked oestradiol to tracer via an intervening molecule of BSA. The proposed advantages of conjugation via BSA are twofold. Firstly, because more than one molecule of tracer may be incorporated into each conjugate molecule, the system results in an "amplification" of E/R-labelling (assuming that only one of the several oestradiol molecules in the conjugate is available for binding to E/R). Secondly, the BSA serves as a protein "carrier" and improves the solubility of the steroid conjugate. Lee (1978) regards this improved solubility as essential in view of the very high steroid concentrations required for his histochemical method. Of those workers who have used BSA-containing conjugates, Lee (1978) and Walker et al. (1980) have employed conjugation at the C<sub>6</sub> position and have used fluorescein or peroxidase respectively as tracers (compounds 3 and 4, Fig. 34). Pertschuk et al. (1979), on the other hand, have employed fluorescein as tracer conjugated via the C<sub>17</sub> position (compound 6, Fig. 35).

In the present study, the effects of conjugation at the C<sub>6</sub> position on affinity of binding to E/R have been investigated (p 139). The results indicate that any "interference" with the C<sub>6</sub> position markedly impairs affinity of binding to E/R, in contrast to the minimal effect of such "interference" on binding to anti-oestradiol antibody (Fig. 36).

The simple addition of a keto group at C<sub>6</sub> reduced the relative binding affinity to 0.13 (similar to the value of 0.22 quoted by Dandliker et al., 1978 for this compound). Conjugation to BSA further reduced the relative binding affinity to 0.09 and the final oestradiol-BSA-peroxidase conjugate, as used by Walker et al. (1980), had a relative affinity of only 0.02.

In contrast to the poor binding affinities of these "C<sub>6</sub>" conjugates, a compound comprising oestradiol, BSA and fluorescein conjugated via the C<sub>17</sub> position prepared by the author had a relative affinity of 0.05 and a similar conjugate donated by Dr. L. Pertschuk had a value of 0.8. This last value is undoubtedly impressive but the point made by Dandliker et al. (1978) regarding the activities of such compounds must be heeded in its interpretation:

"In establishing the activities of fluorescent hormone derivatives, a crucial point must be kept in mind. Assessment of activity by inhibition of [<sup>3</sup>H] oestradiol binding alone is not sufficient clearly to establish activity of the fluorescent conjugate, especially if the relative affinity is low (<2%). In this situation a small amount of an active contaminant or a small amount of degradation resulting in liberation of free, unlabelled hormone could lead to an inflated estimate of the relative affinity."

The possibility that such a situation pertained with regard to the specimen of Dr. Pertschuk's conjugate analysed cannot be excluded.

In summary, labelled conjugates of oestradiol which have been reported to be useful for E/R histochemistry have involved conjugation at either the C<sub>6</sub> or C<sub>17</sub> position, and have involved either direct linkage of oestradiol to the tracer or linkage via a molecule of BSA. Despite the

theoretical advantages of conjugation at  $C_6$ , the binding affinities of such compounds are, in practice, inferior to those of " $C_{17}$ " conjugates. The incorporation of BSA into the conjugate has theoretical advantages in terms of amplification of E/R labelling and improved conjugate-solubility.

#### D. Concentration of Oestradiol Conjugates

In addition to variations in the nature of the oestradiol conjugate selected, as discussed above, the various investigators in the field of E/R histochemistry have also differed with regard to the concentration of conjugate employed. For E/R assay systems in general, it is felt that a concentration of labelled oestradiol which is adequate to saturate the receptor, but which is not vastly in excess of the saturating concentration, should be used. Concentrations of 1 to 5 nM (McGuire et al., 1977; King et al., 1979) have been suggested as suitable for biochemical techniques utilising a single saturating dose of [ $^3\text{H}$ ] oestradiol. If concentrations vastly in excess of the saturating dose are employed, binding to proteins of low affinity is increased, i.e. there is high "non-specific binding". It should be noted that at sufficiently high oestrogen concentrations, binding to serum albumin ( $K_d = 10^{-4} - 10^{-5}$  Molar) and other low affinity binding proteins will be seen. In addition, this binding can be "blocked" by still larger excesses of unlabelled compound. Such binding and displacement are not, of course, related to E/R activity but might be a source of misinterpretation of histochemical appearances.

The concentrations of fluorescent oestradiol (17-FE) of 2 nM and 30 nM used by Dandliker et al. (1978), and by Barrows et al. (1978), are of the same order as the range quoted for biochemical assays and would not, therefore, be expected to give rise to excessive "non-specific binding". Those investigators who have employed albumin-containing conjugates have, however, used much higher concentrations. Pertschuk et al. (1979) recommended a concentration of 1400 nM with respect to oestradiol, equivalent to a conjugate concentration of around 370 nM. Lee (1978 & 1980) used an even higher concentration of 1,000,000 nM with respect to oestradiol, equivalent to a conjugate concentration of around 40,000 nM. Walker et al. (1980), who used a conjugate

similar in structure to that of Lee, did not quote a working concentration but stated that it must be determined empirically by testing various dilutions of the synthesised material.

The conjugate concentrations recommended by the latter groups appear excessive. However, it must be borne in mind that the oestradiol-BSA-fluorescein conjugate used by Pertschuk may have a binding affinity for E/R of only 1/20 of that of oestradiol (estimate on conjugate synthesised by present author). On this basis it might be considered justifiable to use a conjugate concentration 20X as great as that recommended for oestradiol, but Pertschuk's 370 nM is still in excess of this. Similarly, the binding affinity of an oestradiol-BSA-peroxidase conjugate of similar structure to those used by Lee and Walker may be only 1/50 of that of oestradiol 17 $\beta$  (present author's estimate). Even allowing for this, the 40,000 nM conjugate concentration of Lee would seem to be excessive, and might be expected to result in unacceptably high non-specific binding.

A further point to be borne in mind in justification of the high conjugate concentrations advocated by some investigators is that E/R in a solid phase has a lower effective binding affinity for oestrogens than that of the cytosol protein in a liquid phase. This author's experiments with acetone- and protamine-precipitated pellets reflected this apparent alteration in affinity in elevated values of  $K_d$ . Such an alteration in the binding properties of E/R means that a higher steroid concentration is needed to achieve saturation. It also means, however, that the binding properties of E/R are approaching those of non-specific binding proteins.

In summary, the range of concentrations of labelled oestradiol conjugates advocated by various investigators is very wide. Some of the recommended concentrations seem excessive in comparison with those generally recommended for

E/R saturation. Such increased concentrations can, at least in part, be justified on the basis of the relatively low binding affinities of the conjugates and of the apparently impaired binding properties of the E/R protein under histochemical (solid phase) conditions.



## E. Evaluation Of Histochemical Techniques In Practice.

The essential basis of any E/R assay system is the demonstration of tissue uptake of labelled oestradiol which can be blocked by saturation of the receptors with an excess of an appropriate, unlabelled competitor. Failure to demonstrate such blocking in any assay system must raise serious doubts as to whether or not tissue-labelling can be genuinely attributed to E/R activity. With this principle in mind, it may be considered that the following criteria should be noted when examining the results claimed for E/R-histochemistry techniques: firstly, the demonstration of discrete, localised tissue-labelling; secondly, the blocking of such labelling with appropriate competitors and thirdly, correlation with the results of biochemical E/R assays. The results of various investigators, including the author's experiences with two well-documented histochemical techniques, will now be discussed with regard to these criteria.

### a) Studies With Simple Fluorescein-Oestradiol Derivatives

Dandliker et al. (1978) incubated unfixed sections of rabbit uterus with their compound 17-FE and were able to demonstrate discrete fluorescein-labelling, largely localised to the endometrium, at steroid concentrations of 2 - 5 nM. Unfortunately, they were unable to demonstrate blocking of such labelling by co-incubation with unlabelled oestradiol or diethylstilboestrol at a concentration of 1.2  $\mu$ M (approximately 200x excess). In addition, they found that incubation with their compound 6-FE (shown to have a very low binding affinity for E/R), with oestrone labelled with fluorescein at C<sub>19</sub> and with progesterone labelled at C<sub>3</sub>, gave rise to identical staining patterns. As regards correlation with the results of cytosol E/R assays, these authors examined two human mammary tumours, both designated E/R positive by SDG assay. They found that one became .



labelled with 17-FE and that the other did not. I must concur with the conclusion drawn by Dandliker et al. from their results:

"Because many factors may be involved in the staining observed, we cannot at present attribute the staining to any one, such as the localised presence of oestradiol receptors."

More recently, Barrows et al. (1980) reported on studies with a compound designated "FE<sub>2</sub>" which differed from the 17-FE of the previous group in that fluorescein was linked to oestradiol at C<sub>17</sub> via a succinamide-ethyl-amine bridge rather than a carboxy-methyl-oxime bridge. In their studies, Barrows et al. investigated the "temperature-dependent nuclear transfer" of FE<sub>2</sub> as a marker for E/R action rather than the simple cytoplasmic uptake of the labelled steroid. They reported that cytoplasmic labelling occurred in some oestrogen-independent tissues such as liver or gut mucosa but that nuclear-transfer occurred only in target tissues such as endometrium and prostatic and mammary cancers. These investigators reported a measure of success in blocking this nuclear-labelling with appropriate competitors: partial blocking was achieved by pre-incubation with oestradiol at a concentration of 10<sup>-6</sup> M (approximately 300x excess), but for complete blocking a mixture of saturated solutions of diethylstilboestrol, equinilone, oestradiol and oestrone was required! Barrows and co-workers were able to compare the results of their technique with those of cytosol E/R assay in 17 cases of human mammary cancer and report a significant (though far from perfect) correlation.

b) Studies With Albumin-Containing Oestradiol Conjugates

Lee (1978 and 1980) incubated unfixed frozen sections with a C<sub>6</sub>-linked conjugate of oestradiol, BSA and fluorescein. He reported that E/R-negative tissues (appendix, tonsils and carcinomas of stomach and colon) failed to take up the

fluorescent stain but that some cells of human mammary cancers exhibited brilliant, green fluorescence. In common with other investigators, Lee admitted to experiencing difficulty in successfully blocking fluorescent labelling. He maintained that oestradiol is insufficiently soluble in aqueous media to reach concentrations high enough to compete with the 1 mM steroid concentration in his conjugate solution. He was able to achieve blocking by using a conjugate of oestradiol with a BSA "carrier" to improve solubility. Lee admitted, however, that various modifications had to be made to his routine technique to achieve complete blocking: either his normal conjugate concentration had to be reduced by a factor of 10, or a modified conjugate with a low steroid : protein ratio had to be used. In addition, blocking studies were carried out at 4°C rather than the routine room-temperature. It is difficult to accept, in view of these major differences between experimental and control sections, that blocking has been achieved by Lee. In his later report, Lee (1980) claimed an incidence of "E/R-positivity" of 74% as assigned by his technique. This is of the same order as the E/R-positive incidence usually quoted using conventional techniques (Hawkins et al., 1980). With regard to the correlation between histochemical and biochemical estimates of E/R status, Lee (1979) reported on 40 tumours assayed in both ways and found no correlation. In his 1980 report he also stated that there was no direct correlation.

In the technique of Pertschuk et al. (1979), unfixed, frozen sections were incubated with a C<sub>17</sub>-linked conjugate of oestradiol, BSA and fluorescein, then "post-fixed" in ethanol/acetone. They reported the finding of bright, discrete cytoplasmic and/or nuclear fluorescence in mammary cancer specimens and MCF-7 cells after incubation with conjugate. This fluorescence was significantly or completely blocked by co-incubation with 100 - 500x excess of oestradiol, diethylstilboestrol or CI-628. In addition, sections incubated with a "control" conjugate of BSA and

fluorescein exhibited only minimal fluorescence. In two series of 101 and 226 cases respectively (Pertschuk et al., 1979 and 1979<sup>b</sup>), there was a correlation of 82% and of 92% with the results of conventional assays. In the hands of its originators, therefore, this technique would seem to fulfil all the requirements of a satisfactory E/R assay system: discrete tissue-labelling, blocking by appropriate competitors and good correlation with the results of conventional assays.

The attempts of the present author to reproduce these results are presented in "Results XI". Oestrogen-target cells such as those of rat endometrium did exhibit a bright fluorescence after incubation with conjugate, but this labelling could not be blocked by even very high concentrations of competitors. In addition, E/R-negative tissues such as rat duodenum exhibited a very similar staining pattern to that seen in uterus. In these studies, therefore, it would appear that all the tissue-labelling observed could be attributed to non-specific uptake. In view of the comments made above about the effects of post-fixation with ethanol/acetone, a high level of non-specific staining might be expected, on theoretical grounds, using a technique of this type.

Walker et al. (1980) used a C<sub>6</sub>-linked conjugate of oestradiol, BSA and peroxidase on acetone-fixed frozen sections. By this method they were able to demonstrate strongly-staining cells in endometrium and myometrium of rat uterus and also to demonstrate cytoplasmic and/or nuclear staining in some cells of human breast cancers. Sections of rat lung, regarded by these authors as an E/R-negative tissue, failed to exhibit staining. They reported that positive staining could, in all cases, be completely blocked by co-incubation with tamoxifen. The concentration of tamoxifen required was not stated in their publication but a personal communication from Dr. Walker indicated that a saturated solution was used. Results of

the histochemical method were compared with those of DCC assay in 35 cancers and a reasonable correlation was found. Like the technique of Pertschuk, therefore, this method would appear, at least as performed by its originators, to fulfil all the criteria for a valid E/R assay system.

The first problem encountered by the present author during evaluation of this approach was the presence of endogenous peroxidase activity in the tissues under study. In most immunohistological techniques utilising peroxidase as a tracer, it is standard practice to pre-treat tissue sections in a manner designed to destroy endogenous peroxidase activity prior to incubation with peroxidase-labelled antisera. One such method of treatment is to immerse sections in a solution of 3% hydrogen peroxide in methanol. Exposure to such a medium would almost certainly destroy the functional integrity of E/R, which is less robust than the antigenic determinants of most proteins (Walker, personal communication) and it is, therefore, considered impractical to remove endogenous peroxidase from tissues prior to E/R-histochemistry. In the present study a pronounced endogenous peroxidase reaction was observed in human endometrial cells and also in the E/R-negative tissue, rat duodenum. The presence of this staining made it impossible to determine whether or not any additional peroxidase-staining, attributable to specific binding of conjugate, had occurred and would seem to preclude the use of these readily available tissues, of established receptor status, in the validation of this technique.

The experiences of this author with the method of Walker et al. in 25 tissues without detectable endogenous peroxidase staining are presented in Results (147). In agreement with the report of Walker et al. (1980), cellular uptake of conjugate was demonstrable in many biochemically E/R-positive tissues. In this author's hands, however, such uptake was also demonstrable in many tissues which were biochemically E/R-negative. Walker et al. claimed that

blocking of conjugate-uptake was achieved in all cases with tamoxifen. The present author however, in common with many others in the field of E/R-histochemistry, found blocking to be very difficult to achieve and to be only partial in most instances.

Using the demonstration of cellular uptake of conjugate which could be blocked by coincubation with competitor as the criterion for designating a tissue E/R-positive, and taking absent uptake as the criterion for designating a tissue E/R-negative, this author found agreement with DCC assay in only 6 of 25 tissues studied.

In summary therefore, the results of Dandliker et al. (1979), with their compound 17-FE, fail to fulfill the criteria for E/R determination in that blocking of cellular-labelling could not be demonstrated. The results of Barrows et al. (1980) and of Lee (1978 and 1980) may be regarded as more encouraging though their demonstrations of blocking cannot be regarded as entirely satisfactory in that Barrows et al. found it necessary to employ a remarkable "cocktail" of oestrogens and Lee had to modify his assay conditions for the "control" sections. In the hands of their originators, the methods of Pertschuk et al. (1979) and of Walker et al. (1980) would appear promising, but it remains for their successes to be duplicated in other centres. The present author has been unable to reproduce the specificity of staining, the effectiveness of blocking and the excellence of correlation with the results of biochemical assay reported by these groups.



## F. Summary and Conclusions

The studies presented in this thesis allow the following conclusions concerning histochemical techniques for the identification of E/R to be drawn:

1. Losses of E/R activity would seem to occur at every stage of histochemical processing. Reduction in E/R activity has been demonstrated during the actual cutting of frozen sections, during exposure of tissue to fixatives or, in the case of unfixed tissues, during exposure to aqueous media. Overall, it appears that perhaps only 25% of the E/R activity originally present in a tissue remains "viable" for histochemical labelling.
2. From the results of experiments into various methods of E/R immobilisation using the well-established tracer, tritiated oestradiol, it would appear that acetone-fixation gives superior results to air drying, to ethanol/acetone post-fixation and also to the two novel approaches investigated, ammonium sulphate and protamine sulphate precipitation.
3. Various conjugates of oestradiol with tracer molecules suitable for histochemical localisation are able to bind to E/R, as judged by competition studies against tritiated oestradiol. Among the conjugates tested however, the affinity of binding to E/R is only 1/20 to 1/50 of that exhibited by oestradiol 17 $\beta$ .
4. Of the conjugates in current use, those conjugated via the C<sub>17</sub> position on the oestradiol molecule exhibit superior binding to E/R compared with those conjugated via C<sub>6</sub>.
5. It seems necessary to use very high concentrations of competitors to achieve even partial blocking of conjugate-uptake. This raises the concern that such concentrations

might serve to saturate even non-specific oestrogen binding sites and that inhibition of conjugate-uptake by such concentrations may not be truly indicative of bona fide E/R activity.

6. In techniques employing peroxidase as tracer, the presence of abundant endogenous peroxidase in many classical E/R-positive and -negative tissues effectively precludes the use of such tissues for validation of the techniques.

In summary, there is wide agreement that a reliable and reproducible technique for the identification of E/R in tissue sections would represent a significant advance over current cytosol assays. Excellent correlation with the results of biochemical assays have been reported by at least two groups (Pertschuk et al., 1979; Walker et al., 1980). However, the losses of E/R activity inherent in histochemical processing, the poor binding affinities of the oestradiol conjugates used, the inconsistencies between concentrations of conjugates and competitors required and the accepted binding characteristics of E/R and the lack of reports of success with these methods from other centres must raise serious doubts that E/R is truly being demonstrated.

### III. FINAL REMARKS

#### The Prediction Of Response To Endocrine Therapy In Mammary Cancer

Thus far, neither a curative therapy for advanced breast cancer nor an adjuvant therapy of proven worth for primary disease, is available. Endocrine manipulation remains, therefore, a major treatment-option for the palliation of advanced disease and for investigations into the value of adjuvant therapy. The justification for the use of endocrine therapies in such situations is facilitated by the availability of predictive indicators of response. At the present time, E/R status, as assigned by biochemical, cytosol assay, remains the best available predictive indicator. Patients can, with considerable confidence, be excluded from treatment by hormonal means, on the basis of negative E/R status, but the clinical value of positive E/R status is much less. An additional test to identify that subgroup of E/R-positive tumours which are genuinely hormone-dependent is still needed. The test evaluated in this thesis, peroxidase assay, has failed to fulfill its theoretical promise; evidence is accumulating, however, that progesterone receptor (Pg/R) may be a useful additional marker and that patients whose cancers possess both E/R and Pg/R should not be denied hormonal therapy.

In overcoming the limitations of current E/R assay methodology, histological techniques may ultimately prove to be of benefit. The techniques discussed in this thesis depend on the integrity of E/R-oestrogen binding; a property which seems "fragile" and unsuited to study under standard histochemical conditions. It may be, however, that the antigenic determinant of the E/R molecule is more "robust" and, should a specific antibody to E/R become generally available, then the detection of E/R in tissue sections may become a more realistic proposition.



PART V

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PART VIAPPENDIX

ABBREVIATIONS

Standard chemical formulae have been used as required in the text as have established abbreviations such as g (gram), M (Molar), etc. The following standard prefixes have been used in association with such units:

m	:	milli	( $10^{-3}$ )
u	:	micro	( $10^{-6}$ )
n	:	nano	( $10^{-9}$ )
p	:	pico	( $10^{-12}$ )
f	:	femto	( $10^{-15}$ )

Other abbreviations have been defined on the first occasion on which they appear in the text. For completeness and ease of reference, an alphabetical list of abbreviations used is presented here:

A/R	:	Androgen receptor
BSA	:	Bovine serum albumin
cpm	:	Counts per minute (as a measure of radioactivity)
DAB	:	Diaminobenzidine tetrahydrochloride (a histochemical stain for peroxidase)
DCC	:	Dextran coated charcoal (used in E/R assays)
DFI	:	Disease-free interval
DMBA	:	7,12 dimethyl benz(a)anthracene (used for the induction of rat mammary tumours)

DPX	:	Standard histological mounting medium
E <sub>2</sub>	:	Oestradiol 17 $\beta$
<sup>o</sup> E <sub>2</sub>	:	"Unlabelled" Oestradiol 17 $\beta$ as used in competition binding assays
[ <sup>3</sup> H] E <sub>2</sub>	:	[2,4,6,7 - <sup>3</sup> H] Oestradiol 17 $\beta$ (Tritium-labelled E <sub>2</sub> for E/R assay)
EC	:	Enzyme Commission
E/R	:	Oestrogen Receptor
FDNB	:	1-fluoro-2,4,-dinitrobenzene
FITC	:	Fluorescein isothiocyanate
HRP	:	Horseradish peroxidase
K <sub>d</sub>	:	Dissociation constant
K <sub>m</sub>	:	Michaelis constant
MW	:	Molecular weight
P <sub>o</sub>	:	Receptor site concentration, usually in fmoles/mg wet weight
PBS	:	Phosphate buffered saline (0.3 M, pH 7.6) standard buffer for histo-chemistry
Pg	:	Progestogen
<sup>o</sup> Pg	:	"Unlabelled" progestogen (Organon 2058) as used in competition binding assays
[ <sup>3</sup> H] Pg	:	Tritium-labelled Progestogen (Organon 2058) for Pg/R assay
Pg/R	:	Progestogen receptor

rpm : Revolutions per minute

S : Svedberg unit, a measure of sedimentation rate

SDG : Sucrose Density Gradient (a separation technique used in E/R assays)

**Short Communication**

**ENDOGENOUS PEROXIDASE: AN ALTERNATIVE TO OESTROGEN RECEPTOR IN THE MANAGEMENT OF BREAST CANCER?**

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MANY WORKERS have shown that human breast cancers which contain oestrogen receptors (RE) are more likely to respond to hormonal therapy than those which do not (McGuire *et al.*, 1978). RE status, as determined by conventional techniques is a relatively poor indicator of responsiveness to hormonal therapies; only 35–63% of patients classified as RE<sup>+</sup> respond to endocrine ablation (Roberts *et al.*, 1978; McGuire *et al.*, 1978). Standard RE assay techniques require up to 300 mg of tumour, an amount which is often unavailable in cases presenting early with small primary tumours, or where metastatic deposits are inaccessible to open biopsy (*e.g.* in bone or liver).

These limitations reduce the clinical usefulness of RE assays and have led to a search for alternative indicators of hormone responsiveness which might discriminate better and be detected in smaller tumour samples. Progesterone receptor (RP) (McGuire *et al.*, 1978) and endogenous peroxidase (Lyttle & De Sombre, 1977; Duffy & Duffy, 1977; De Sombre *et al.*, 1975) are two proteins which have been suggested as potential indicators of hormone responsiveness.

The present study was set up to assess the value of endogenous peroxidase as an indicator of hormone dependence by measuring peroxidase levels in rat mammary tumours which serve as models of hormone-dependent and -independent growth.

DMBA-induced rat mammary tumours are considered to serve as models of ovary-

dependent growth. Previous work in our department has shown that over 80% of such tumours regress after oophorectomy (Hawkins *et al.*, 1978; Scott *et al.*, 1979). Twenty-four such tumours were examined in this study. Two transplantable lines (TG3 and TG5) of rat mammary tumour which exhibit ovary-independent growth (Hawkins *et al.*, 1978) have been generated in our department and 20 tumours of these lines were also examined.

Each of the 44 tumours was dissected free from the host animal, after exsanguination. The tumour was then homogenized in an ice-cooled tube, at a concentration of 300 mg/ml in cold 10mM Tris buffer (pH 8.0) with 10% glycerol (v/v) using a Silverson homogenizer. The homogenates were centrifuged for 45 min at 39,000 *g* to yield a clear cytosol. A random sample of 12 cytosols from each group of tumours was then processed by mixing each with 100  $\mu$ l of monothio-glycerol and assaying for RE and RP by saturation analysis techniques as described elsewhere (Hawkins *et al.*, 1977, and in preparation). The cytosols from the other tumours were discarded.

Each centrifugation pellet was then homogenized at 300 mg/ml in 10mM Tris buffer (pH 7.4) containing 0.5M calcium chloride to solubilize any peroxidase present. The homogenate was centrifuged for 45 min at 39,000 *g* to yield a clear cytosol containing the solubilized peroxidase. This extraction procedure is based on that of De Sombre & Lyttle (1978).

The cytosol was then assayed for per-



oxidase by a method based on that of Himmelhoch *et al.* (1969). The reaction mixture in the cuvette comprised 13mm guaiacol, 0.4mm hydrogen peroxide and 10mm Tris buffer (pH 7.4) containing 0.5mm calcium chloride, in a final volume of 3 ml. The reaction was started by addition of cytosol, a volume between 0.1 and 0.5 ml being required to give a suitable deflection of the spectrophotometer needle. The rate of reaction was measured by the change in absorbance at 470 nm between 1 and 3 min after starting the reaction.

Receptor concentrations were expressed in fmol/mg of wet tumour and peroxidase content in u/g wet tumour. One peroxidase unit was defined as the amount of enzyme required to produce an increase of one absorbance unit per minute under the assay conditions used.

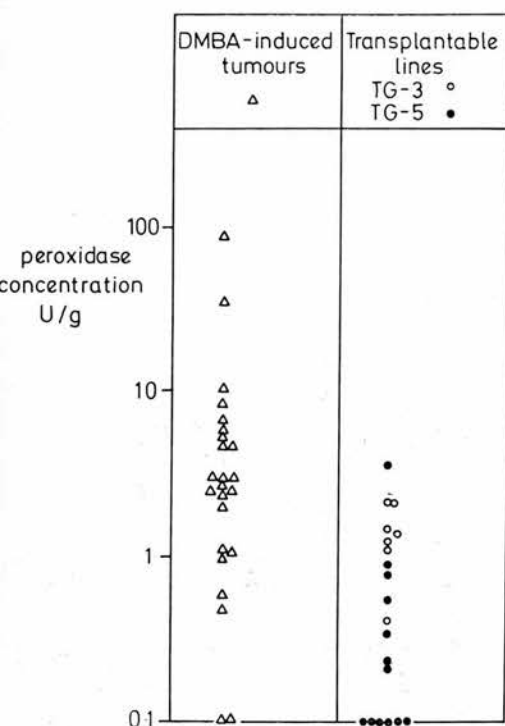


FIG. 1.—Endogenous peroxidase content in rat mammary tumours which serve as models for hormone-dependent and independent growth. Each symbol represents one tumour.

The Wilcoxon rank-sum test was used to evaluate the differences between "hormone dependent" and "hormone-independent" tumours for concentrations of each "indicator-protein".

Fig. 1 shows the endogenous peroxidase content of each of the 24 DMBA-induced tumours and each of the 20 transplantable tumours. Peroxidase was detectable in all but 2 of the DMBA-induced tumours, but was undetectable in 6 of the transplantable tumours. Analysis of the levels in the two groups revealed a highly significant difference ( $P < 0.001$ ). Nevertheless there is a considerable overlap in peroxidase levels between the "hormone-dependent" and "independent" tumour models.

Figs. 2 and 3 show the levels of RE and RP in a random sample of 12 tumours from each group. Significant differences were again found between "hormone-dependent" and "independent" tumours. In the case of RE there was no overlap between the groups. RP was undetectable in one DMBA-induced tumour, but other-

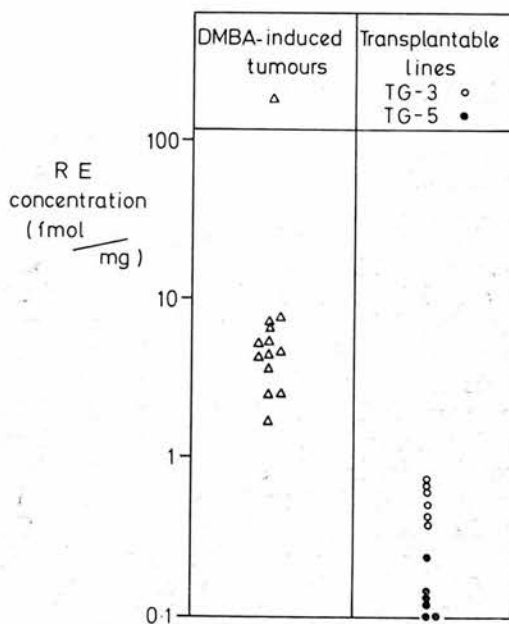


FIG. 2.—Oestrogen receptor content in rat mammary tumours which serve as models for hormone-dependent and independent growth. Each symbol represents one tumour.

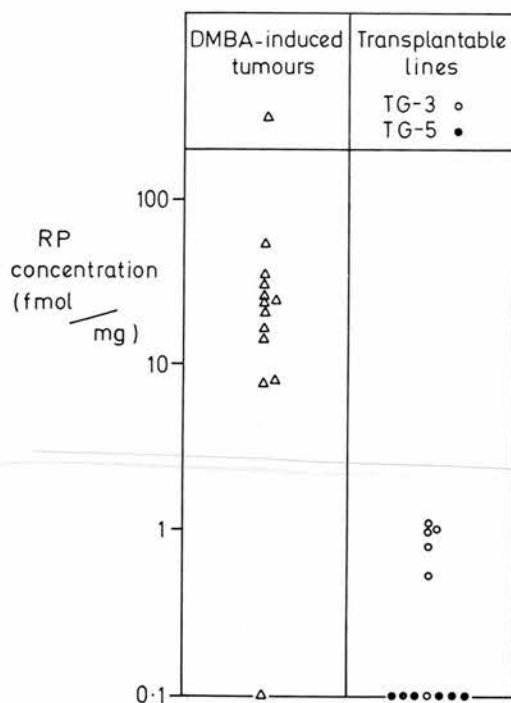


FIG. 3.—Progesterone receptor content in rat mammary tumours which serve as models for hormone-dependent and independent growth. Each symbol represents one tumour.

wise the range of levels in the two groups was quite distinct.

The results obtained in this study indicate that RE and RP assays clearly discriminate between hormone dependence and independence in these rat mammary tumours. It is known that not all DMBA-induced tumours are hormone-dependent and it might be postulated that the one such tumour in which no RP could be detected might have belonged to the hormone-independent minority of DMBA-induced tumours. This study revealed no overlap in RE levels between the two groups but it should be noted that in earlier generations of the TG3 and TG5 lines higher RE concentrations were found, and the distinction between the groups was less clear-cut (Hawkins *et al.*, 1978; Scott *et al.*, 1979).

Our results support the postulated relationship between peroxidase content and

hormone dependence. However, the overlap in peroxidase levels between the largely hormone-dependent, DMBA-induced group and the hormone-independent transplantable group was considerable. It seems unlikely, therefore, that endogenous peroxidase will prove to be a more reliable discriminator than RE.

Our findings are somewhat at variance with those of Lyttle and his co-workers (1979) in their studies of peroxidase levels in mouse mammary-tumour models. They found no overlap between hormone dependent and independent groups.

Peroxidase can be readily identified by a simple histochemical technique (De Sombre *et al.*, 1975) and could, therefore, be detected in much smaller biopsy specimens than are needed for standard RE assays. Peroxidase estimations in breast tumours might, therefore, prove clinically useful in overcoming one of the limitations of standard receptor assays—namely, the amount of tumour needed. However, the findings of this study suggest that peroxidase is unlikely to provide a more accurate prediction of hormone-responsiveness than RE.

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